Molecular mechanisms of shift in steroidogenesis during meiotic maturation and testicular recrudescence in teleost fish models

Thesis submitted for the degree of

Doctor of Philosophy

By

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CERTIFICATE

This is to certify that **Mr. G. Sreenivasulu** has carried out the research work embodied in the present thesis under my supervision and guidance for a full period prescribed under the Ph.D. ordinance of this University. We recommend his thesis "**Molecular mechanisms of shift in steroidogenesis during meiotic maturation and testicular recrudescence in teleost fish models**" for submission for the degree of Doctor of Philosophy of this University.

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I hereby declare that the work embodied in this thesis entitled "**Molecular mechanisms of shift in steroidogenesis during meiotic maturation and testicular recrudescence in teleost fish models**" has been carried out by me under the supervision of Prof. B. Senthilkumaran and this has not been submitted for any degree or diploma of any other university earlier.

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To my mother

Ovary, oogenesis

Oogenesis in its broadest sense is the process by which primordial germ cells become ova that are ready for fertilization. Oogenesis is fundamentally similar in vertebrates and differences between the taxa are superficial. Oogenesis can be broadly divided in to six major steps: (1) formation of primordial germ cells (germline segregation), (2) transformation of primordial germ cells into oogonia (sex differentiation), (3) transformation of oogonia into oocytes (onset of meiosis), (4) growth of oocyte while under meiotic arrest (vitellogenesis), (5) resumption of meiosis (maturation), and (6) expulsion of ovum from its follicle (ovulation; Patino and Sullivan, 2002).

Inspite of great diversity with an equally diverse array of reproductive strategies, teleost ovaries show a general structure (Nagahama, 1983). Ovaries of adult fish are generally paired structure surrounded by conjuctiva tunica and mesovarium attached to the body cavity on either side of dorsal mesentery. The posterior part of each ovary is prolonged by an oviduct connected to genital papilla. Ovaries are compartmentalized by numerous septa formed by folds of the germinal epithelium, called ovigerous lamellae, projecting into the ovarian lumen. These lamellae contain nest of oogonia, oocytes at early stages of entry into the meiotic prophase-I and follicles at various stages of oocyte and follicle growth and differentiation. In contrast to mammals, teleost oogonia keep on proliferating in adult females thus renewing the stocks of oocytes and follicles (Tokarz, 1978). Apart from this general scheme, there are some exceptions. Viviparous poecilids have only one ovary (Dodd, 1986) which presents specialized structures devoted to long

term preservation of spermatozoa (Jalabert, 2005). On the contrary, the ovaries of salmonids such as trout and salmon are not completely surrounded by the mesovarium and ovigerous lamellae that are open to the body cavity where mature oocytes are directly released during ovulation. At times, they remain for a while before being laid down through genital papilla (Jalabert, 2005).

The functional unit within the ovary is ovarian follicle, which has remarkably similar architecture in most teleosts. The developing oocyte is located in the centre of follicle and is surrounded by follicular cell layers. The granulosa and thecal layers are separated by a basement membrane. Between the surface of oocyte and granulosa layer there is an acellular layer, zona radiata the future egg chorion in which the microvilli originating from both oocyte and granulosa layer forms pore canals. Cytoplasmic gap junctions between granulosa cells and oocyte have been shown to form essential conduits between them for the transfer of nutrients and chemical messengers (Nagahama, 1983; Kessel *et al*., 1985; York *et al*., 1993). Granulosa cells form a regular monolayer, but the morphology and functions of these cells vary between animal and vegetal poles (Iwamatsu *et al*., 1994). Highly specialized cells at animal pole, the micropylar cell, closes up the micropyle and appears as a kind of funnel with a small opening on the oocyte side which will be just enough to allow a sperm to get through (Riehl, 1974). In comparison with a pre-ovulatory mammalian follicle, fish post-vitellogenic follicle has no antrum and most of its volume is occupied by oocyte with enormous amount of yolk and a large germinal vesicle (Jalabert, 2005).

Oocyte growth - Vitellogenesis

Following the onset of meiosis, significant growth of the oocyte begins after formation of ovarian follicle (folliculogenesis) and the structure of follicle remains essentially unchanged throughout follicular growth (Wallace and Selman, 1990; Patino and Takashima, 1995; Patino and Sullivan, 2002). Follicular growth generally occurs in two stages, pre-vitellogenic and vitellogenic. At the onset of pre-vitellogenic growth, oocytes arrest their meiosis at the diplotene stage of prophase-I. Large amounts of ribosomal RNA and much of the mRNA present in full-grown oocytes are produced during pre-vitellogenic growth. The content of certain mRNAs, such as vitellogenin receptor (VgR) and vitellogenin (Vg) processing enzyme mRNAs thought to peak during pre-vitellogenic growth (Wallace and Selman, 1990). Large amounts of glycoproteins are also synthesized and incorporated into alveoli (cortical alveoli) at the oocyte's periphery. Deposition of lipids derived from circulating very low density lipoprotein generally begins during pre-vitellogenic growth (Prat *et al*., 1998).

Vitellogenic growth is characterized by accumulation of exogenously synthesized yolk proteins within the oocyte (Wallace, 1985). Vg is a large glycophospholipoprotein (300-600 kDa) synthesized by liver, transported via the blood stream to ovary and taken up by growing oocytes (Patino and Sullivan, 2002). Using tracer technique, it has been demonstrated that Vg enters the ovarian follicle through capillaries of thecal layer. Then it passes through the basement membrane, the intercellular spaces of granulosa cells, finally through the pore canals of vitelline envelope and makes contact with oocyte

surface (Fig. 1). Vg binds to a specific VgR on the oocyte surface and internalization of Vg-VgR complex occurs at specialized areas of the oocyte membrane called coated pits (Stifani *et al*., 1990). Proteolytic cleavage of Vg into yolk proteins, lipovitellin and phosvitin occurs in the internalized vesicular bodies and lysosomal cathepsin D acts as yolk protein generating enzyme (Hiramatsu, 2002). Yolk proteins are stored as yolk globules or platelets throughout the ooplasm (Blazer, 2002).

Natural induction of Vg synthesis in liver ultimately ensues from activation of the hypothalamic-hypophyseal-gonadal axis in response to endogenous as well as environmental cues. Reports in teleosts indicate that increased levels of plasma follicle stimulating hormone (FSH) induce follicular eastradiol-17β (E_2) production, which in turn, stimulates hepatic Vg synthesis (Fig. 1; Specker and Sullivan, 1994). In some species, ovarian E_2 production may be regulated by luteinizing hormone (LH; Okuzawa, 2002). Estrone may contribute to induction of Vg by priming the liver to respond more strongly to E_2 (van Bohemen *et al*., 1982). Besides gonadotropins, several other hormones such as melatonin, thyroxine, insulin and growth hormones have been implicated in regulating vitellogenesis (Wallace, 1985; Senthilkumaran and Joy, 1995; Patino and Sullivan, 2002).

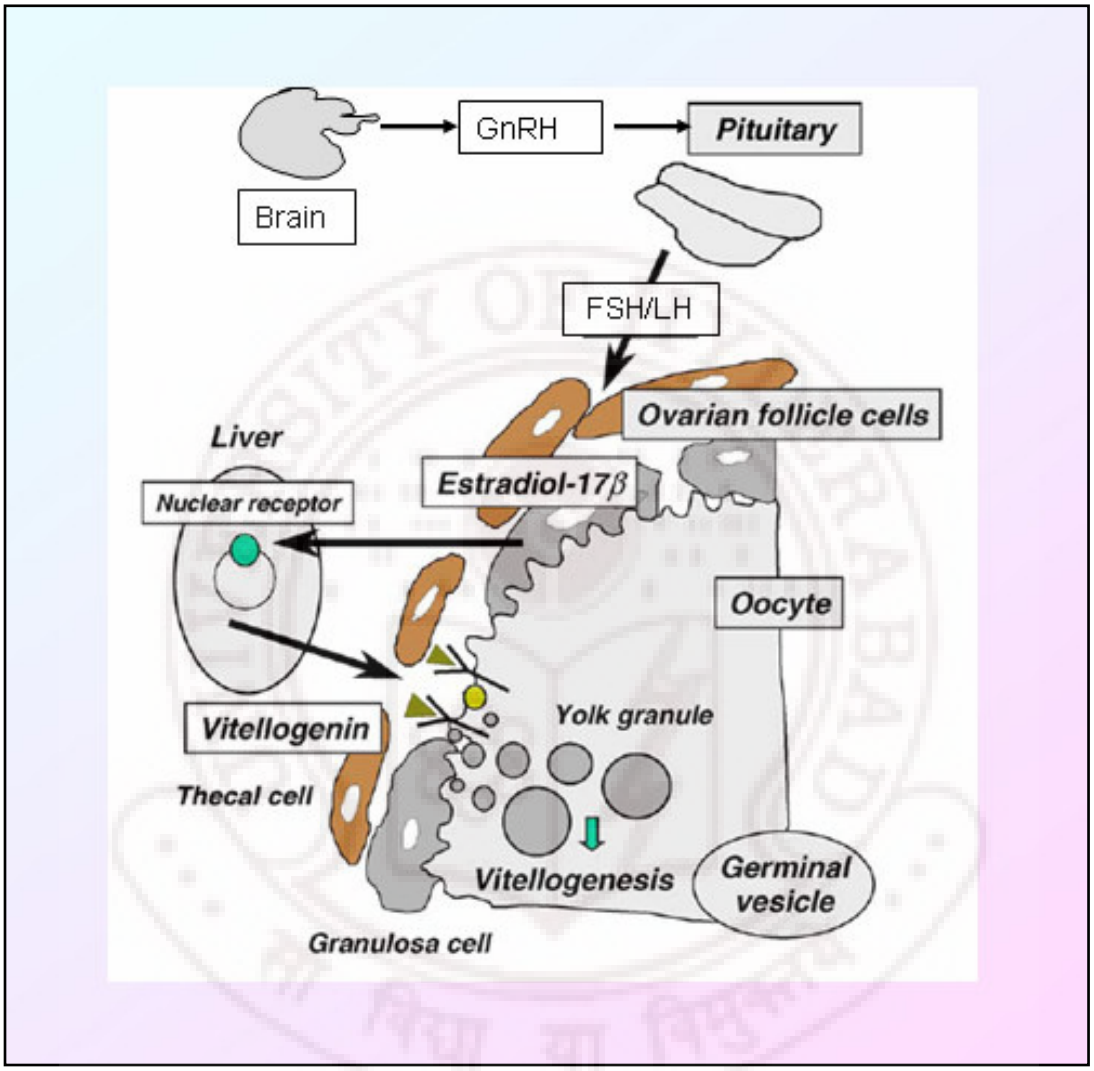

Fig. 1. Schematic representation of the hormonal regulation of vitellogenesis (adopted from Senthilkumaran *et al*., 2004).

Final oocyte maturation (FOM)

In many teleosts, plasma LH levels begin to rise (well known as pre-ovulatory LH surge) after the completion of vitellogenesis (Joy *et al*., 1998; Khan and Thomas, 1999). LH binds to its receptor on granulosa cells and stimulates a sequence of events including acquisition of maturational competence, production of maturation-inducing hormone (MIH), MIH-dependent resumption of oocyte meiosis and cytoplasmic maturation. Upon completion of the first meiotic division and release of first polar body, meiosis in the matured oocyte is arrested again at metaphase-II that is resumed during fertilization (Nagahama, 1994).

In general, the most obvious morphological change of FOM is migration of germinal vesicle towards animal pole and is often used as an indicator (Upadhyaya and Haider, 1986; Senthilkumaran and Joy, 2001). This process involves changes in cytoskeletal networks such as microtubule distribution (Lessmann *et al*., 1988). Following migration, several other processes occur in the nucleus and cytoplasm including germinal vesicle breakdown (GVBD) that indicates the completion of prophase-I, condensation of chromosomes, formation of spindle and expulsion of first polar body indicating the completion of meiosis-I (Nagahama, 1994). Cytoplasmic changes observed during GVBD include coalescence of lipid droplets and yolk globules with increased oocyte translucency (Goetz, 1983). In some teleosts, especially marine species, GVBD is accompanied by marked hydration involving Na^+/K^+ -ATPase

dependent ion regulation, resulting in the swelling of oocyte (LaFleur and Thomas, 1991).

Oocyte maturational competence

Maturational competence refers to the ability of folliculated oocyte to resume meiosis when stimulated with MIH (Patino *et al*., 2001). LH-dependent acquisition of maturational competence requires activation of protein kinase A pathway (Chang *et al*., 1999), *de novo* protein synthesis and is also associated with increased mRNA levels of gap junction proteins, oocyte membrane MIH receptor (Thomas *et al*., 2001 & 2004) and formation of homologous and heterologous gap junctions. Insulin-like growth factor (IGF) and activins may modulate or mediate LH-dependent maturational competence in teleost ovarian follicles. In red seabream, IGF-I is a strong inducer of maturational competence (Kagawa *et al*., 1994) as well as gap junction contacts (Patino and Kagawa, 1999). IGF-I also induces membrane MIH receptor activity (Thomas *et al*., 2001). Activins A and B induce maturational competence in ovarian follicles of zebrafish and co-treatment with follistatin suppresses it (Pang and Ge, 2000a).

Three stage concept of oocyte maturation

Several studies both in teleosts and amphibians, demonstrated that oocyte maturation is regulated by three major mediators, namely, gonadotropin (GTH), MIH and metaphase promoting factor (MPF; Nagahama, 1994 & 1997; Planas *et al*., 2000; Senthilkumaran

et al., 2004; Nagahama and Yamashita, 2008; Fig. 2). Gonadotropic control of oocyte maturation has been best studied in fishes. Pituitary GTH induces the production of MIH by ovarian follicle cells in a two-step process (Nagahama, 1994). First, GTH induces the production of 17α-hydroxyprogesterone (17α-OHP) in thecal cells. Secondly, this precursor steroid is converted to 17α, 20β-dihydroxy-4-pregnen-3-one (17α, 20β-DP) by the granulosa cells.

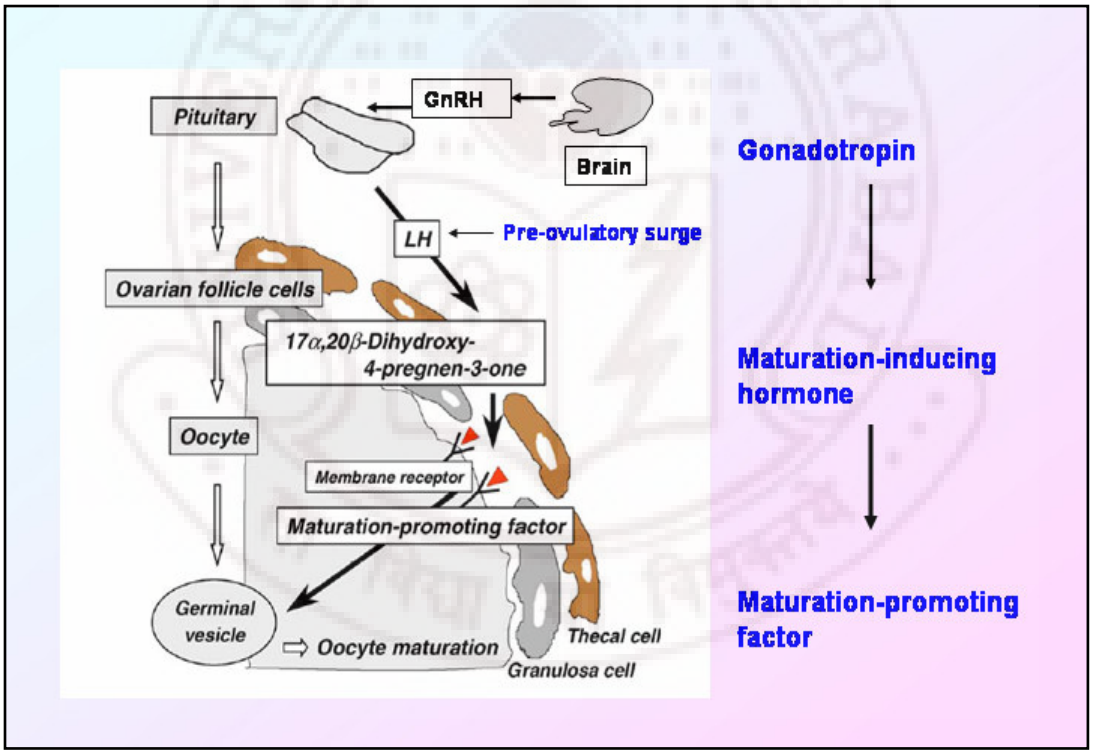

Fig. 2. Schematic representation of hormonal regulation and three stage concept of oocyte maturation (adopted from Senthilkumaran *et al*., 2004).

Identification of MIH

Numerous studies have been conducted to test the effects of various steroids on the induction of FOM *in vitro*. Among the C18, C19 and C21 steroids tested so far, the C21 steroids have been shown to induce FOM more potently than other two groups of steroids. 17α, 20β-DP has been first identified as amago salmon MIH (Nagahama and Adachi, 1985). Subsequent reports designated it as MIH for several teleosts. Trant and Thomas (1989) identified a related MIH, 17α, 20β, 21-trihydroxy-4-pregnen-3-one (20β-S), as a potent inducer of GVBD in a marine perciform, the Atlantic croaker. There is strong evidence that MIH is a steroid, specifically progesterone or its derivatives in fish species studied to date (Jalabert *et al*., 1991). However, androgen has been shown to be produced by *Xenopus* oocytes and induce FOM potently (Lutz *et al*., 2001). Apart from these, corisol and catecholestrogens were also reported to induce FOM in Indian catfishes (Goswami and Sundararaj, 1974; Senthilkumaran and Joy, 2001). Although MIH is generally considered as mediator of LH-induced meiotic resumption, a number of other factors are also known to mediate or modulate this process. IGF-I (Kagawa *et al*., 1994; Weber and Sullivan, 2000 & 2001), activins (Ge, 2000; Wu *et al*., 2000), epidermal growth factor, transforming growth factor (Pang and Ge, 2002b) and others have been shown to induce FOM. Serotonin seems to negatively modulate MIH-dependent meiotic maturation in ovarian follicles of *Fundulus heteroclitus* (Cereda *et al*., 1998). Arachidonic acd (AA) and its metabolic products appear to regulate an unidentified step in the biochemical pathway leading to meiotic

maturation in ovarian follicles of the European seabass (Sorbera *et al*., 2001). However, neither AA nor its metabolites seem to influence MIH-dependent oocyte maturation in ovarian follicles of the Atlantic croaker (Patino *et al*., 2003).

Mechanism of synthesis of MIH: Two-cell type model

Using incubation of isolated follicular preparations, a two-cell type model has been proposed, for the first time in any vertebrate, for the production of MIH (Nagahama, 1994 & 1997). In this model, the thecal cell layers produce 17α-OHP from progesterone by the action of the enzyme 17α-hydroxylase. 17α-OHP traverses the basal lamina and is converted to 17α, 20β-DP in the granulosa cells where GTH acts to enhance the activity of 20β-hydroxysteroid dehydrogenase (20β-HSD), the key enzyme required for the conversion of 17α-OHP to 17α, 20β-DP (Fig. 3; Young *et al*., 1986; Nagahama, 1987). The GTH stimulation of thecal cells to produce 17α-OHP involves receptor-mediated activation of adenylate cyclase and formation cAMP. The GTH action on 20β-HSD enhancement in granulosa cells has been found to be mimicked by forskolin, dbcAMP and by two phsopodiesterase inhibitors (Nagahama *et al*., 1985a; Kanamori and Nagahama, 1988). Subsequently, *in vitro* experiments using both transcriptional and translational inhibitors have demonstrated that GTH and cAMP induction of 20β-HSD activity involves new RNA and protein synthesis (Nagahama *et al*., 1985b).

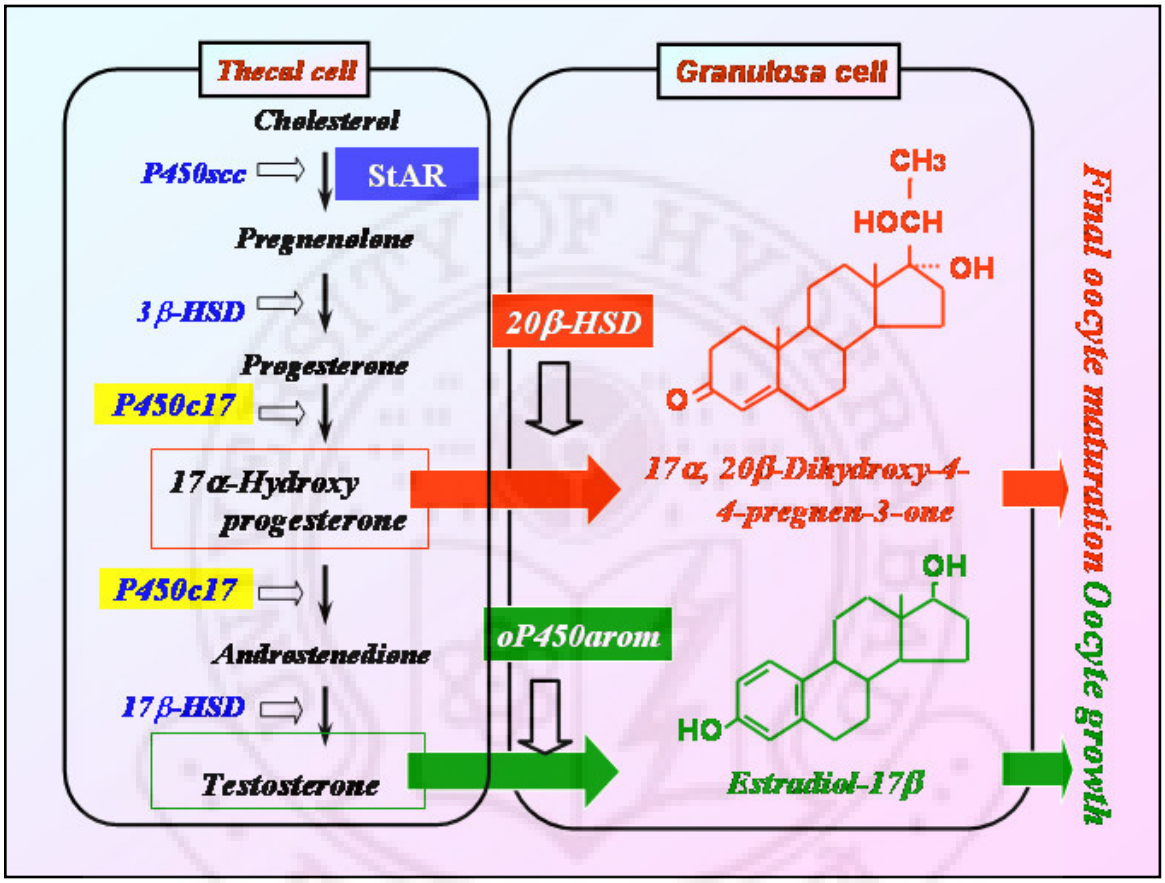

Fig. 3. Schematic representation of two-cell type model in the shift in steroidogenesis occurring in fish ovarian follicles prior to oocyte maturation (adopted from Senthilkumaran *et al*., 2004).

Shift in steroidogenesis occurring in ovarian follicles prior to oocyte maturation

The ability of ovarian follicles to produce E_2 in response to GTH increases during oocyte growth, but rapidly declines with the onset of FOM in response to GTH (Kagawa *et al*., 1983; Joy *et al*., 1998; Kumar *et al*., 2000). It is also reported that testosterone production by thecal layer preparations during oocyte growth and maturation gradually increases in response to GTH and peaks during post-vitellogenic period (Kanamori *et al*., 1988). This capacity of thecal layers is maintained during the period of oocyte maturation and ovulation. Ovarian aromatase (oP450arom), enzyme that produces E_2, activity in granulosa cells increases during vitellogenesis and decreases rapidly with the onset of FOM in response to GTH (Yoshiura *et al*., 2003). This decrease in oP450arom activity seems to be coincident with decreased ability of follicles to produce E_2. On the other hand, immediately prior to oocyte maturation, ovarian follicles acquire increased capability to produce 17α, 20β-DP. Granulosa cells acquire the ability to convert 17α-OHP to 17α, 20β-DP well before the onset of maturation and an increase in 20β-HSD activity was noticed during FOM (Senthilkumaran *et al*., 2002). However, thecal cells do not develop the capacity to produce 17α-OHP until they receive a signal from GTH during oocyte maturation (Kanamori *et al*., 1988). Therefore, a decrease in C17-20 lyase activity and an increase in 20β-HSD activity appear to be the major factors responsible for massive production of 17α, 20β-DP during FOM (Nagahama, 1994 & 1997). Though it cannot be

generalized, the two-cell type model seems to hold good for many teleost species (Fig. 3).

Mechanism of action of MIH

Following synthesis by follicle cells, MIH travels across the zona radiata to reach the oocyte and induce FOM. Granulosa cells and oocyte are physically coupled through their microvillar-gap junctions and this coupling is known to be regulated developmentally and hormonally. Using chemical inhibitors of gap junctions, it is now established that the gap junctions are essential conduits for intra-follicular transport of MIH to oocyte in vertebrates (Patino and Purkiss, 1993; York *et al*., 1993).

In fishes, microinjection of MIH into full-grown immature oocytes was ineffective in inducing GVBD while external application was effective suggesting the presence of MIH receptors on oocyte membrane. Progesterone, a MIH in amphibians apparently acted via membrane receptors (Fig. 2; Nagahama, 1997). Further, membrane receptors for 17α, 20β-DP and 20β-S have been found in different fish species (Patino and Thomas, 1990; Yoshikuni *et al*., 1993; Thomas *et al*., 2004) and subsequently cDNA clones were obtained in fishes as well as in other vertebrates (Zhu *et al*., 2003a & b). A significant increase in 17α, 20β-DP and 20β-S receptor concentrations was noticed in ovaries of flounder and seatrout undergoing FOM. Consistently, *in vitro* treatment of ovarian follicle with GTH also raised the concentration of MIH membrane receptors.

This GTH induced elevation in membrane MIH receptor coincides with development of oocyte maturational competence (Patino and Thomas, 1990).

The mechanism by which the binding of MIH to its receptor induces FOM was controversial. Based on the studies from fishes as well as from other vertebrates, utilizing phsophodiesterase inhibitors such as 3-isobutyl-1-methylxanthine and theophylline and adenylate cyclase activators such as forskolin and choletera toxin, it has been found that the 17α, 20β-DP induced FOM involves a transient decrease in oocyte cAMP level and subsequently cAMP signaling cascade involving protein kinase A (Jalabret and Finet, 1986; Haider, 2003). Further, using pertusis toxin, inhibitory G protein has been shown to involve in mediating the action of 17α, 20β-DP (Yoshikuni and Nagahama, 1994; Nagahama, 1997). On the other hand, a decrease in membrane diacyl glycerol and protein kinase C signaling is also thought to be involved in 17α, 20β-DP action (Smith, 1989).

MPF

A key event during 17α, 20β-DP induced oocyte maturation is formation and/activation of MPF in the cytoplasm of oocyte. Identification and purification of MPF in unfertilized amphibian eggs was a land mark in cell biology research (Masui and Clarke, 1979; Lokha *et al.*, 1988). In contrast to GTH and MIH, MPF displays no species-specificity and acts not only in inducing meiotic maturation but also as a universal regulator of G2/M phase transition in the cell cycle of various organisms

ranging from yeast to mammals (Nagahama and Yamashita, 2008). In all animals examined to date, including fishes, MPF is composed of two protein subunits: a protein having MPF kinase activity that is homologous to $cdc2^{+}$ gene product of fission yeast referred to as p^{34cdc2} (cdc2) and a regulatory protein cyclin B (Yamashita, 1998).

In general, MPF activity is controlled by phsophorylation and dephosphorylation of cdc2 on Thr14, Tyr15 and Thr161 after a complex formation with cyclin B. The cdc2 activity requires Thr161 phosphorylation, which is catalyzed by cyclin dependent kinase (CAK) consisting of cdk7 and cyclin H. phosphorylation of Thr14 and Tyr15 by Weel/Myt1 inactivates cdc2 even if Thr161 is phosphorylated. Dephosphorylation of Thr15/Tyr15 is catalyzed by cdc25 (Coleman and Dunphy, 1994; Morgan, 1995; Nurse, 2002).

Two models concerning activation of MPF have been proposed and investigated extensively in several vertebrate species. First, a constant level of cdc2 is maintained during oocyte maturation and cyclin B protein is newly synthesized from its stored mRNA in oocyte after a signal from MIH (Yamashita *et al*., 2000). The newly synthesized cyclin B immediately forms a complex with pre-existing cdc2, rendering the CAK to phosphorylate cdc2 on Thr161 thus leading to the activation of MPF. Since CAK can not phosphorylate monomeric cdc2, complex formation with cyclin B seems to be compulsory. Thus, synthesis of cyclin B protein in response to MIH is necessary and sufficient to induce oocyte maturation in goldfish (Katsu *et al*., 1993). This proposition was confirmed by the finding that MIH-induced GVBD is blocked by the

anti-sense RNA mediated cyclin B synthesis in the Japanese brown frog, *Rana japonica* (Ihara *et al*., 1998).

Secondly, immature oocytes of *Xenopus* and starfish consists inactive MPF called pre-MPF (Yamashita *et al*., 2000). Here in this case, in pre-MPF, Thr161 and Thr14/Tyr15 of cdc2 are in phosphorylated state and since dephsophorylation of Thr14/Tyr15 is necessary to make MPF active. Hence, the action of MIH (progesterone in *Xenopus*) is to activate cdc25 that impairs the ability of Myt1 to phosphorylate Thr14/Tyr15 of cdc2 which leads to the activation of MPF (Smith and Nebreda, 2002; Haccard and Jessus, 2006).

The model proposed for goldfish is known to operate in several other fish species too including carp, catfish, zebrafish and also in lamprey giving the impression that absence of pre-MPF is common to fish (Yamashita *et al*., 2000). But later on, in fresh water perch (Basu *et al*., 2004) and trout (Qiu *et al*., 2007) pre-MPF has been found in immature oocytes and the activation of MPF as in *Xenopus* seems to be the key event to induce oocyte maturation in these species although this contention needs further clarification. In between these goldfish and *Xenopus* models, the presence of low levels of pre-MPF has been identified in the axolotl (Vaur *et al*., 2004) immature oocytes. Slow and progressive dephsophorylation of Thr14/Tyr15 of cdc2 is the turning point in this species. However, in spite of differences in the formation and activation of MPF from species to species, the molecular structure and functions are common in all eukaryotes (Yamashita *et al*., 2000; Nurse, 2002).

Testis, spermatogenesis

A great diversity do exists in the morphology of testis. In agnathans, testicular tissue is present in a single cord and in hagfish it is common for this cord to contain ovarian tissue too (Gorbman, 1983). Germ cells develop in follicles which eventually rupture to release sperm into body cavity. In elasmobranches, testes are paired structures within which germ cells and their supportive Sertoli cells divide and develop together in discrete spherical units called spermatocysts (ampullae; Dodd, 1986). Teleost testis is typically paired and has a lobular or tubular organization within which germ cells develop. During this process they were partially embedded in Sertoli cells (Nagahama, 1983). The mitotic spermatogonia are organized in lobules along entire length of tubular segment and the mature sperm are released into central lumen which eventually leads to efferent ducts followed by urogenital opening. Sperm production in some species is a single synchronous event, while in others it is cyclic or even continuous.

Despite great diversity in testis types, it contains germ cells in synchronous or variable stages of development and a complement of somatic cells specialized for physical support and regulation of spermatogenesis, including Sertoli and Leydig cells. Sertoli cells are found in direct association with germ cells which they support physically and nurture by modifying the chemical microenvironment. Leydig cells are typically interspersed in the connective tissue and their primary function is to produce steroids needed for spermatogenesis (Nagahama, 1983).

Fish spermatogenesis is fundamentally similar to that of vertebrates (Nagahama, 1983; Callard, 1991). Differences between taxa are superficial. Characteristic cytological changes during spermatogenesis include mitotic proliferation of committed spermatogonial stem cells and the subsequent differentiation of some, but not all, into primary spermatocytes. The first meiotic division marks the conversion of primary spermatocytes into secondary spermatocytes which in turn undergo a second meiotic division to form spermatids. During spermiogenesis the spermatids mature, develop a flagellum and separate from their supportive Sertoli cells to become spermatozoa (Schulz and Miura, 2002).

Culture of testicular fragments with GTH or other steroids induced the entire process of spermatogenesis within 24 days (Miura *et al*., 1991). The human chorionic gonadotropin (hCG)-induced spermatogenesis *in vitro* activates Sertoli and Leydig cells markedly in terms of ultrastructural changes and active production of steroids. Addition of hCG to culture media containing testicular fragments induced a rapid increase in 11-ketotestosterone (11-KT) which is known to be a potent androgen in most of the fishes, whereas in *Fundulus*, only testosterone promoted the spermatogenesis *in vitro* (Cochran, 1992). However, it is believed that the action of 11- KT is mediated by other factors produced by Sertoli cells, where the presence of androgen receptor is detected (Ikeuchi *et al*., 2001). It is possible that some of these factors are growth factors such as IGF-I and activin B. Further, up-regulation of activin B cDNA has been demonstrated within the first 24 hours of hCG-treatment *in vivo* in eels (Miura and Miura, 2003). All

these studies indicate that the hormonal regulatory mechanisms for spermatogenesis include GTH induced spermatogonial proliferation and testicular 11-KT production which in turn activates the Sertoli cells to produce activin B. Activin B then acts on spermatogonia to induce mitosis leading to the formation of spermatocytes (Fig. 4; Miura and Miura, 2003).

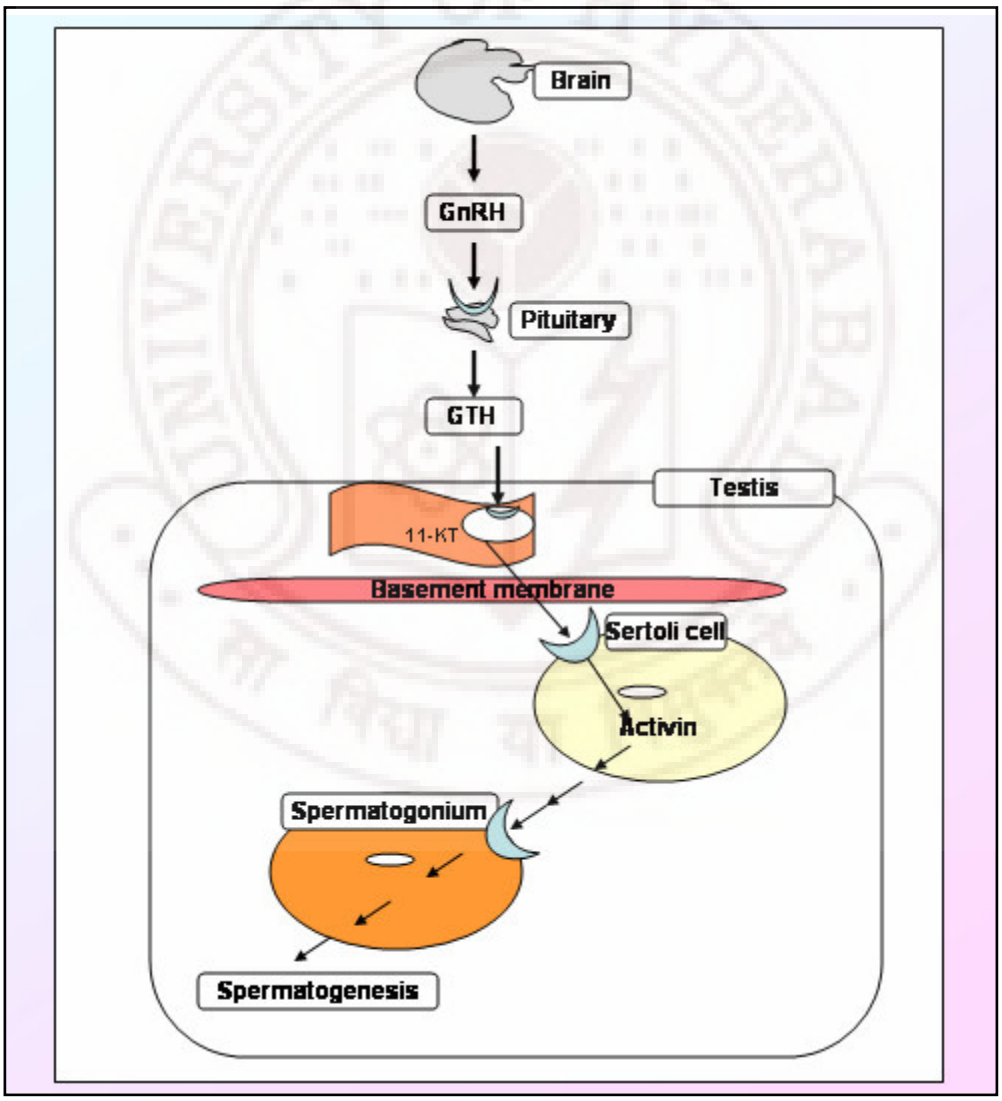

Fig. 4. Schematic representation of hormonal regulation of spermatogenesis in fish.

Sperm maturation

In fish, spermatozoa in the sperm duct are immotile and acquire motility when confronted with water suggesting that after completing spermiogenesis, spermatozoa attains the ability of motility. Sperm maturation is the phase during which non-functional gametes develop into mature spermatozoa, which are fully capable of motility and fertilization. Sperm maturation seems to involve only physiological changes but not morphological changes. In salmonids, sperm maturation has been induced by increasing the seminal plasma pH to 8.0 (Miura *et al*., 1992) in the sperm duct. Later on, it has been found in eel also (Miura *et al*., 1995; Ohta *et al*., 1997). Sperm maturation is also under endocrine regulation and in some teleosts 17α, 2β-DP is known to induce sperm maturation (Miura *et al*., 1992). However, the action of 17α, 2β-DP is not direct on sperm, rather it increases seminal plasma pH, which in turn increases the sperm cAMP content thereby allowing the acquisition of sperm motility (Miura, 1995). Using subtractive cDNA library approach, a cDNA named as eel spermatogenesis related substance 22 has been cloned that is homologous to carbonic anhydrase (CA; Miura *et al*., 2002). CA catalyzes the reversible hydration of carbon and is involved in ion and acid-base regulation in various tissues and fluids. Subsequently, a progesterone receptor on spermatozoon membrane was identified and activation of CA by 17α, 2β-DP through membrane progesterone receptor is thought to increase the pH of sperm duct (Fig. 5; Nagahama, 1994; Todo *et al*., 2000; Miura and Miura, 2003).

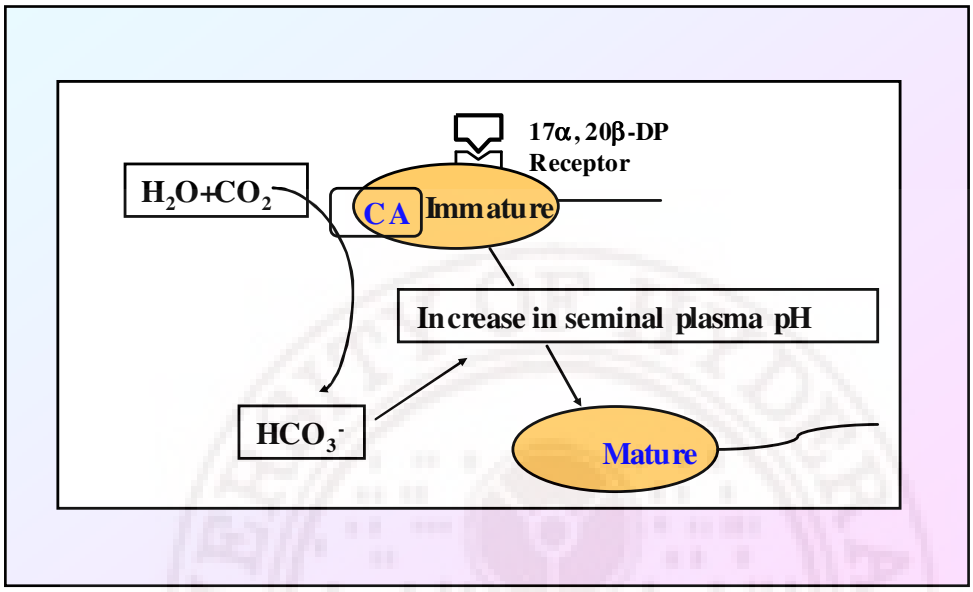

Fig. 5. Schematic representation of sperm maturation in teleosts.

Molecular mechanism of steroidogenic shift-Scope and objectives

Shift in steroidogenesis can happen at two stages, first being the shift in synthesis of precursor steroid from testosterone to 17α-OHP while the second is shift in final steroid products from E_2 to MIH (Nagahama and Yamashita, 2008). The switch in production of theses steroids is likely to be regulated by changes in abundance of steroidogenic enzymes that produce them. Hence, characterization in terms of form and function, expression of steroidogenic enzyme genes gained momentum soon after the initial identification of shift in plasma steroid levels.

Shift in precursor steroid synthesis

Since MIH is synthesized from the 17α-OHP, there should be a shift in synthesis of androstenedione from 17α-OHP to maintain the levels of 17α-OHP. This can occur with the down regulation of C17-20 lyase activity and up-regulation or maintenance of hydroxylase activity. Regulation of P450c17 is complex because it is a single enzyme catalyzing both the reactions. A similar kind of shift in steroid synthesis i.e. from E_2 to progesterone also occurs in mammalian ovary during the transition from follicular phase to luteal/maturation phase. But the production of progesterone takes an alternative pathway (Δ^4) utilizing 3β-hydroxysteroid dehydrogenase (3β-HSD) only and P450c17 is not required in this case. However, the differential regulation of P450c17 is seen in adrenal steroidogenesis during aging and the selective production of corticosteroids and sex steroids are brought about by the differential regulation of C17-20 lyase and hydroxylase activities. Ser/Thr phosphorylation through a cAMP dependent kinase is known to selectively elevate C17-20 lyase activity while dephosphorylation by protein phosphatase 2A keeps hydroxylase activity high. Moreover, redox partners such as cytochrome b5 and P450 oxidoreductase are known to modulate lyase activity. In fish there are no studies to explain the differential actions of P450c17 during steroidogenic shift and it has been thought that similar mechanism that operates in mammalian adrenals also occurs in fish ovary. But recently, a second novel form of P450c17 was identified in tilapia and medaka and has been shown that the differential expression patterns of these enzymes are important for shift in steroidogenesis (Zhou *et al*., 2007a

& b). However, none of the studies in fish are comprehensive to relate the mRNA expression to enzyme activity.

Another possibility for the production of high amount of 17α-OHP during oocyte maturation is increase in the abundance of steroidogenic acute regulatory protein (StAR) and steroidogenic enzymes such as cytochrome P450 side chain cleavage enzyme (P450scc) and 3β-HSD. Possibility of involvement of StAR and above mentioned enzymes is reasonable because (i) many of the steroidogenic enzyme genes and StAR are influenced by the stimulatory effect of tropic hormones, in most cases with an increase in intracellular cAMP levels (ii) stimulation of adenylate cyclase activity and production of cAMP in follicle layers during GTH induced oocyte maturation and finally (iii) *in vitro* experiments with both RNA and protein synthesis inhibitors have suggested the involvement of *de novo* RNA and protein synthesis. Though cDNAs encoding *StAR*, *P450scc* and *3β-HSD*s have been cloned from different fish species, little is known about role in shift in steroidogenesis. In addition, there are no precise studies utilizing *in vitro* and *in vivo* models to implicate a role for these proteins in steroidogenic shift.

Proteins are denoted with normal font while genes with italics throughout the thesis.

Shift in final steroid production

Expression of *oP450arom* gradually increased throughout vitellogenesis and became undetectable in post-vitellogenic ovarian follicles/during meiotic maturation (Watanabe *et al*., 1999). Further, *in vitro* incubation of tilapia post-vitellogenic follicles with hCG-purged *oP450arom* transcripts (Yoshiura *et al*., 2003). Further studies on this line confirmed that AD4BP/SF-1 and FOXL-2 regulates the expression *oP450arom* (Yoshiura *et al*., 2003; Wang *et al*., 2007).

20β-HSD cDNA was first cloned from pig testis (Tanaka *et al*., 1991), since purified protein was available only in pig (Nakajin *et al*., 1988). Surprisingly, Tanaka *et al*. (1991) found that the pig testicular *20β-HSD* cDNA has striking homology (about 85%) to that of human carbonyl reductase1 (CBR1) and shown to be structurally and functionally similar to mammalian CBR1. Since 20β-HSD has broad substrate specificity and low Km for endogenous compounds, involvement of this enzyme in very specific events such as FOM is questionable. Nevertheless, several reports demonstrated the involvement of *20β-HSD* and CBR1 in oocyte maturation and ovulation respectively (Espey *et al*., 2000; Tanaka *et al*., 2002). Further, increase in *20β-HSD* mRNA is known in ayu and trout (Guan *et al*., 1999; Tanaka *et al*., 2002) while sudden appearance was observed in the Nile tilapia during FOM (Senthilkumaran *et al*., 2002). In contrast, a stable expression pattern of *20β-HSD* was noticed in hCG-induced maturation of zebrafish ovarian follicles (Wang and Ge, 2002). Unlike *oP450arom*, transcriptional regulation of *20β-HSD* is least studied (Senthilkumaran *et al*., 2001 & 2004). Since,

expression of both *20β-HSD* and *oP450arom* in granulosa cells is modulated by GTH via cAMP, understanding how cAMP regulates the up-regulation of *20β-HSD* and down regulation of *oP450arom* at a time in the same cell is interesting.

In light of the above reviewed literature, present thesis work is an effort to understand the molecular mechanism underlying the shift in steroidogenesis more explicitly. Following set of objectives (see below) were designed using snake head murrel, *Channa striatus* and air-breathing catfish *Clarias gariepinus* as experimental models. Murrel is a delicious fresh water teleost species of India and is being used in alternate medical practice for the treatment of asthma in Hyderabad (India). Air-breathing catfish is domesticated species in southern parts of India and has high growth rate. The synchronous development of ovary, possibility of collection of matured eggs after inducing FOM with hCG, *in vivo* without sacrificing animals and availability of data on plasma steroid levels during ovarian development (Joy *et al*., 1998; Kumar *et al*., 2000) are some of the characteristic features of catfish model.

Objectives

1. Molecular cloning, characterization, localization and expression of *20β-HSD* during oocyte maturation.
2. Isolation of promoter region of *20β-HSD* and understating the transcriptional regulation.
3. Precursor steroid production for shift in steroidogenesis: Role of *P450c17*.
4. Precursor steroid production for shift in steroidogenesis: Role of *StAR*.

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Abstract

Partial cDNAs encoding carbonyl reductase1-like 20β-hydroxysteroid dehydrogenase (*20β-HSD*) and P450 17α-hydroxylase/c17-20 lyase (*P450c17*) were isolated from the ovary of snake head murrel and they exhibited high sequence identity to the Nile tilapia and rainbow trout respectively. A low transcript level of both *20β-HSD* and *P450c17* were detected in pre-vitellogenic follicles, while the transcript level was high in full-grown immature follicles. In human chorionic gonadotropin (hCG)-induced *in vitro* oocyte maturation, we found a significant increase in *20β-HSD* transcript level after 2 hrs. The *P450c17* transcripts also showed a considerable increase following hCG-induction compared to saline-treated controls. On the other hand, Western blot analysis demonstrated no significant change in the P450c17 protein level during hCG-induced *in vitro* oocyte maturation. Taken together, we suggest that in addition to *20β-HSD*, the *P450c17* might have a role in the shift in steroidogenesis during meiotic maturation of snake head murrel.

Introduction

Oocyte maturation is induced by a maturation-inducing hormone (MIH), secreted by ovarian follicle layers under the influence of gonadotropin. The 17α, 20β, dihydroxy-4-pregnen-3-one was identified as MIH in several teleost species (Nagahama, 1997). The MIH is synthesized from the precursor17α-hydroxyprogesterone by 20β-hydroxysteroid dehydrogenase (*20β-HSD*) and P450 17α-hydroxylase/c17-20 lyase (*P450c17*) is the enzyme that produces 17α-hydroxyprogesterone from progesterone. Studies from several fish species indicated a shift in steroidogenesis from estradiol-17β to 17α, 20β-dihydroxy-4-pregnen-3-one that occurs prior to oocyte maturation (Nagahama, 1997). Elevation of 20β-HSD activity and transcript level in response to gonadotropin induction has also been demonstrated during oocyte maturation (Senthilkumaran *et al*., 2004; Nagahama and Yamashita, 2008). However, the molecular mechanism underlying the steroidogenic shift is unclear with reference to precursor steroids. Hence, the objective of the present study is to investigate the role of *P450c17* in steroidogenic shift. As a first step, partial cDNAs encoding *20β-HSD* and *P450c17* were obtained from the ovary of snake-head murrel, *Channa striatus* using RT-PCR. Expression of *20β-HSD* and *P450c17*, after gonadotropin induction as well as in pre-vitellogenic and full-grown immature ovarian follicles was done. In addition, P450c17 protein level following gonadotropin induction was studied.

Materials and methods

Adult snake-head murrel were bought from the local market in Hyderabad and fish were sacrificed by decapitation. Ovaries were dissected out and total RNA was isolated using TRI Reagent (Sigma, St. Louis, MO, USA) according to manufacturer's instructions. First strand cDNA was prepared with 5µg of total RNA using Superscript III (Invitrogen, Carlsbad, CA, USA). Degenerate primers were designed aligning existing sequence data base (Table 1) and used in RT-PCR amplification of cDNA. RT-PCR products were cloned into pGEM-T Easy (Promega, Madison, WI, USA) vector and sequenced. For human chorionic gonadotropin (hCG)-induction studies, gravid female snake-head murrel were obtained during April/May and maintained in the laboratory until use. Full-grown oocytes with centrally located germinal vesicle were collected by dissecting the ovaries. Oocytes were incubated in triplicate in fish ringer solution containing 100 IU/ml hCG. After the addition of hCG, oocytes were collected at 0,1,2,4,6,8,12,24 and 36 hours and total RNA was prepared. Using gene specific primers, expressions of *20β-HSD* and *P450c17* after hCG-induction were analyzed by semi-quantitative RT-PCR following the method of Kwon *et al*. (2001). The SDS-PAGE was carried out for a 10% homogenate of follicles collected at different time points and transferred to a nitrocellulose membrane. The P450c17 protein was immuno-detected using specific P450c17 heterologous (developed against eel P450c17) polyclonal antiserum.

Primer	Sequence (5' – 3')	Purpose
20β-HSD-DF1	CAGAGTGGTGGATGTVTCHAGC	Degenerate PCR
20β-HSD-DR1	CCTGCCATGTCRGTKCKGACC	Degenerate PCR
P450c17-DF1	GTGGAYACMGTKGCMAAGG	Degenerate PCR
P450c17-DR1	CACYTCYCTGATRGTGGCYTC	Degenerate PCR
20β-HSD-F	GGTTCCATCGCCCTGAGC	RT-PCR
20β-HSD-R	AGGCATTGCACAGGATTTCA	RT-PCR
P450c17-F	TGGATACAGTGGCAAAGGATAGTT	RT-PCR
P450c17-R	GGGTCTGTCCTCCAATCTTAG	RT-PCR
β-Actin-F	ACCGAAGTCCATCACAATACCAGT	RT-PCR
β-Actin-R	GAGCTGCGTGTTGCCCCTGAG	RT-PCR

Table 1. List of primers used for different purposes.

Results

A partial cDNA fragment of 313bp (104 amino acid residues, Fig. 1, 2A) encoding carbonyl reductase1 like-*20β-HSD* was isolated from the ovary of murrel and it showed about 61% homology to its counter part from the Nile tilapia. The *P450c17* partial cDNA obtained was 463bp (154 amino acid residues, Fig. 1, 2B) and exhibited about 83% homology to rainbow trout.

Semi-quantitative RT-PCR analysis demonstrated that the expression of both *20β-HSD* and *P450c17* was low in pre-vitellogenic follicles and abundant in full-grown immature follicles (Fig. 3).

A significant increase in the *20β-HSD* transcript level was detected 2hrs after the hCG-induction (Fig. 4A). An increase in the *P450c17* expression was also found following hCG-induction compared to saline-treated controls (Fig. 4B). Immunoblot analysis of P450c17 protein level at different time points following hCG-induction indicated no considerable change in the P450c17 protein level (Fig. 5). All the experimental results (both semi-quantitative RT-PCR and western blotting) were checked with intensity analysis (data not shown) to depict significant increase of various correlates in hCG-treated group when compared to saline-treated control group.

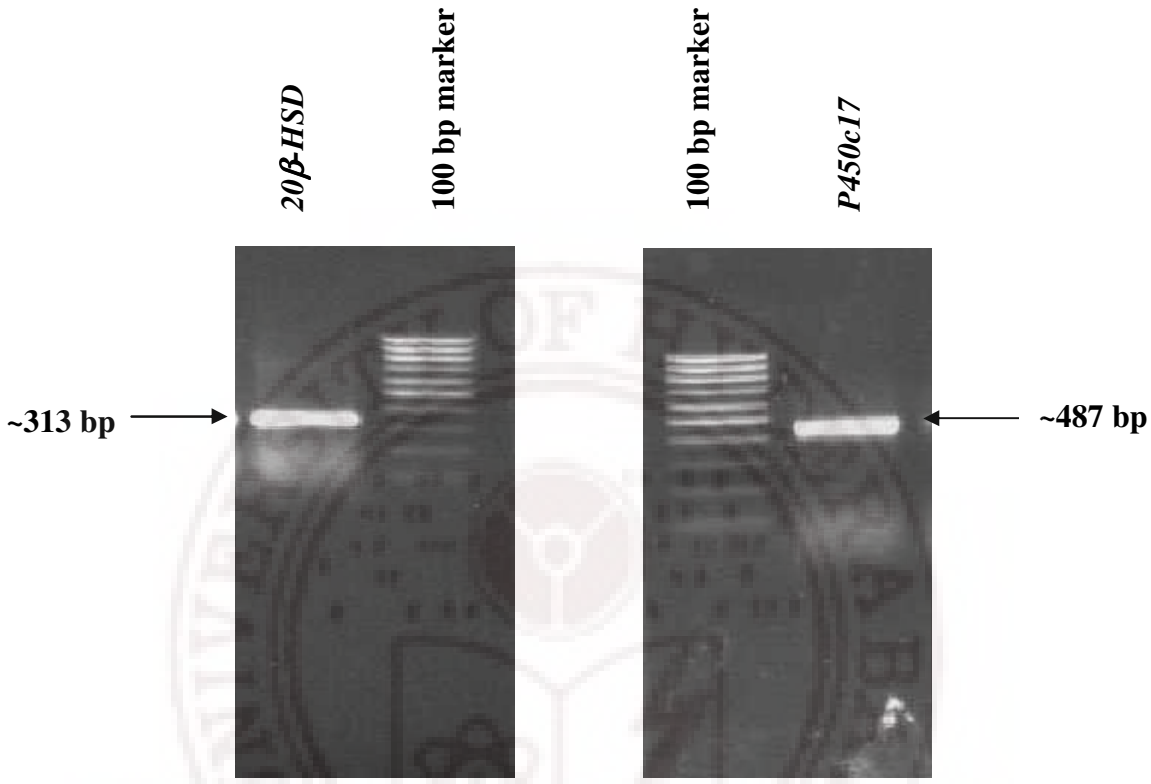

Fig. 1. 1% Agarose gels showing RT-PCR products using degenerate primers for amplification of partial *20β-HSD* and *P450c17* cDNAs.

(A)

RVVNVSSFVSVQALNKCSPALQQRFRSEDITEEELVGL MQKFIDDAKKGEHKQAGWPDTAYGASKLGLTTLSMI LARGLSKERPNDGILLNACCPGWVRTDMAG

(B)

IVDTVAKDSLVDIFPWLQFFPNADLRRLKWCVFIRDQL LQKKYNEHKAHYSDHVQRDLLDALLRAKRSAENNNT AEISAESVGLSDDHLLMTVGDIFGAGVETTTAVLKWA VTYLMHYPEVQRRIQAELDSNIAGDRTPQLSDRGSLLY LEATI

Fig. 2. Deduced amino acid sequence (partial) of snake head murrel ovarian 20β-HSD (A) and P450c17 (B).

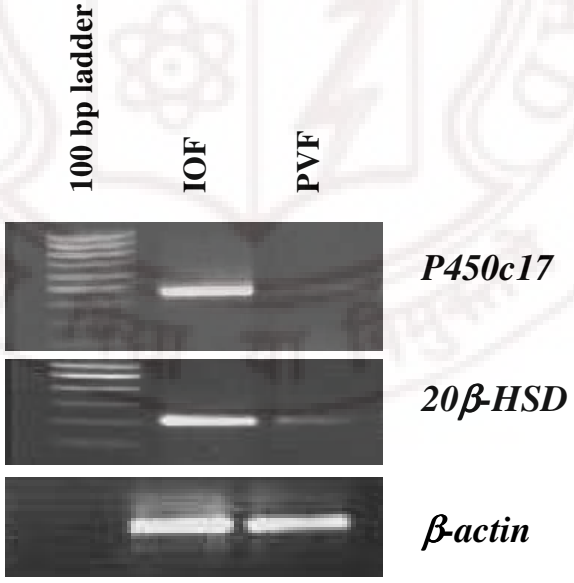

Fig. 3. Semi-quantitative RT-PCR analysis of *20β-HSD* and *P450c17* expression in full-grown immature follicles (IOF) and pre-vitellogenic follicles (PVF) of murrel (representative gel, n=5).

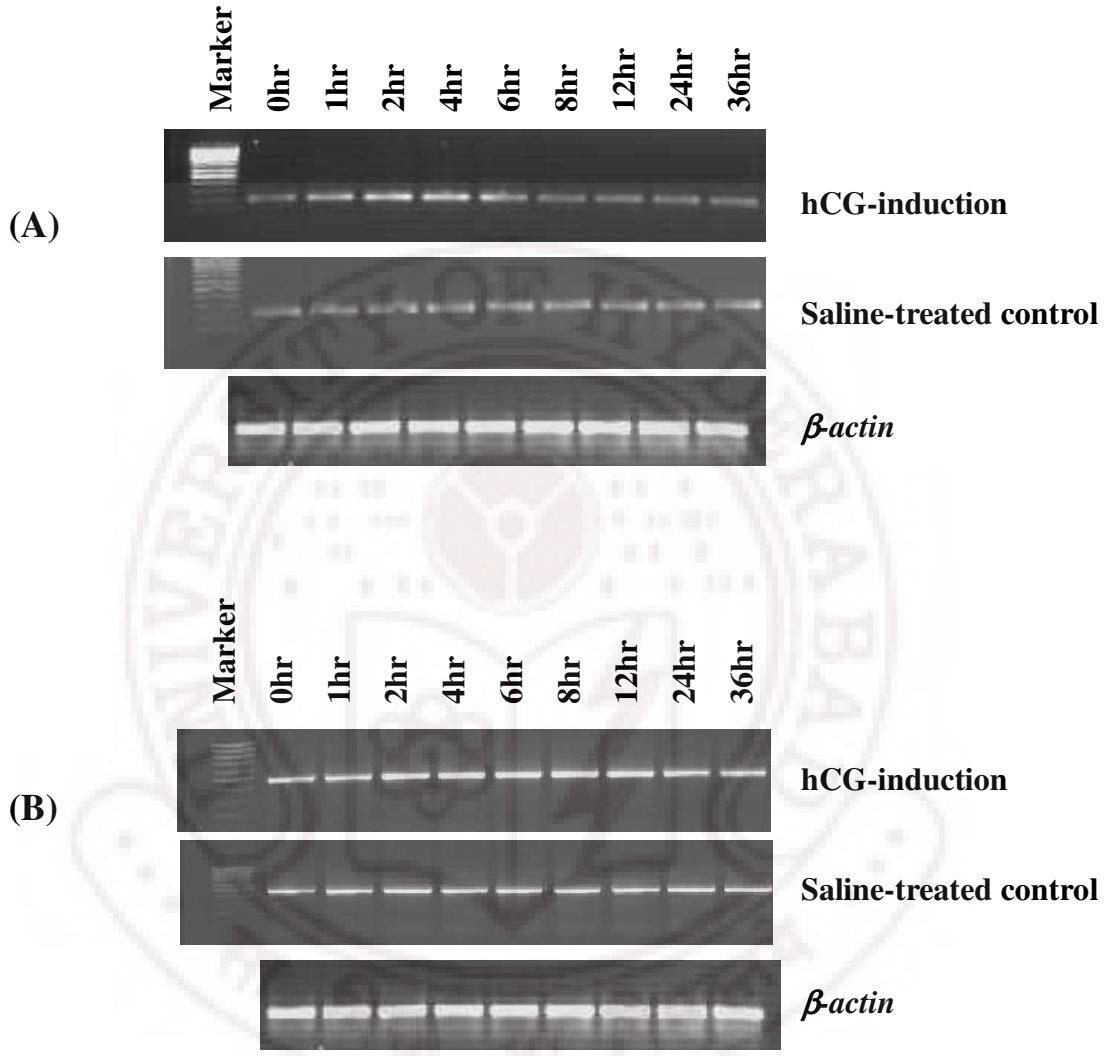

Fig. 4. Semi-quantitative RT-PCR analysis of transcript level of *20β-HSD* (A) and *P450c17* (B) following hCG-induced *in vitro* oocyte maturation at different time periods (representative gel, n=5). Marker used was 100 bp ladder.

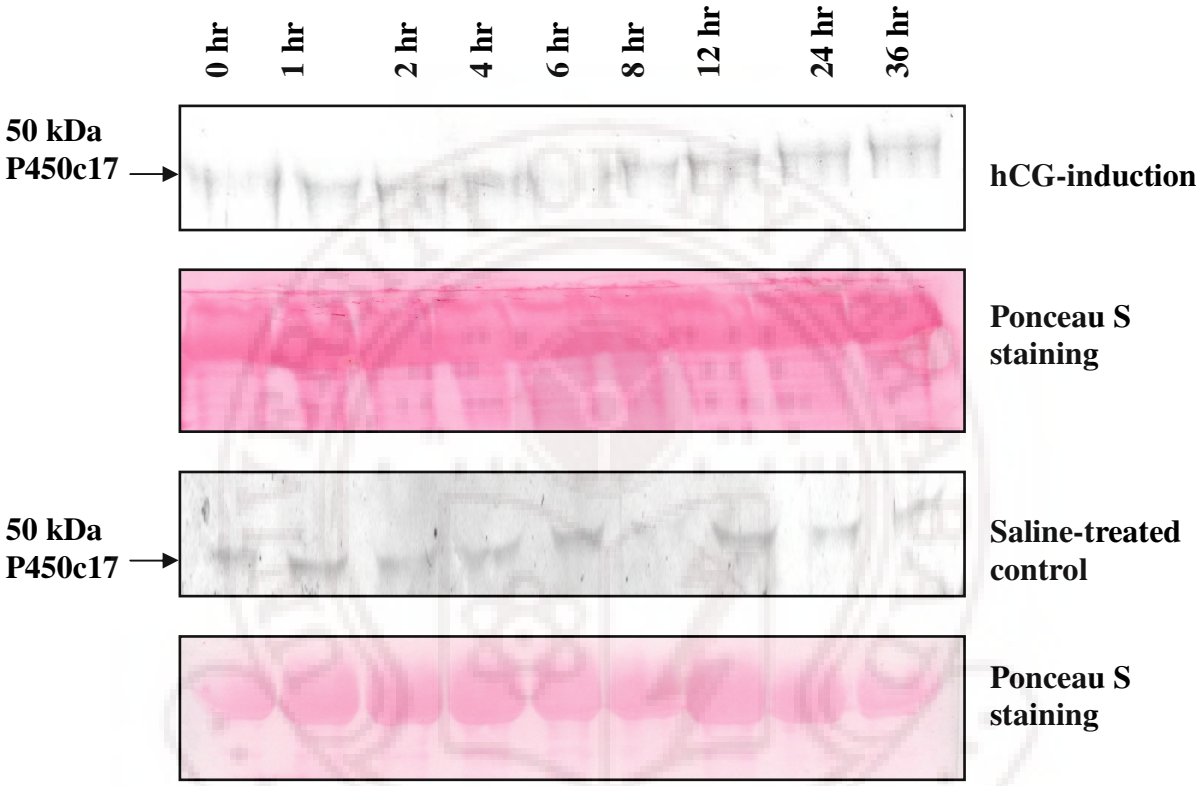

Fig. 5. Western blot analysis of P450c17 during hCG-induced *in vitro* oocyte maturation (representative gel, n=5). Ponceau S staining was used to depict equal loading.

Discussion

Present study was aimed to check the simultaneous expression patterns of *20β-HSD* and *P450c17* during meiotic maturation. The *20β-HSD* obtained from murrel ovary seems to be specific for perciform species as it has high homology to tilapia. The cloned murrel *20β-HSD* and *P450c17* partial cDNA fragments contained the major conserved functional and signature domains. Both *20β-HSD* and *P450c17* transcripts were low in pre-vitellogenic follicles and abundant in full-grown immature follicles. Our observations are consistent with reports in ayu *20β-HSD* (Tanaka *et al*., 2002) and channel catfish *P450c17* (Kumar *et al*., 2000). In hCG-induced *in vitro* oocyte maturation, *20β-HSD* transcript level significantly increased after 2hrs. Earlier study in ayu also demonstrated similar pattern (Tanaka *et al*., 2002). Thorough monitoring of hCG-induced *20β-HSD* denoted steady decline after initial burst of transcriptional activity. Present study was first of its kind to track the expressions of *20β-HSD* as well as *P450c17* throughout meiotic maturation. In the Nile tilapia, an undetectable expression in full-grown immature follicles and a rapid induction following gonadotropin induction has been demonstrated (Senthilkumaran *et al*., 2002). On the other hand, Wang and Ge (2002) reported no significant change in the *20β-HSD* expression during gonadotropin-induced oocyte maturation in zebrafish. Based on our results from murrel and existing reports on other teleosts, we suggest that in annually spawning fishes *20β-HSD* transcript level increases gradually from pre-vitellogenic

follicles to full-grown immature follicles. A small increase in *20β-HSD* transcript level might be sufficient for the shift in steroidogenesis that is required for oocyte maturation. Further, we also found an increase in *P450c17* transcript level following hCG-induced meiotic maturation. This, in addition to *20β-HSD* elevation, made us to speculate that the increased *P450c17* might contribute to steroidogenic shift by providing precursor steroid. With these findings, we analyzed the P450c17 protein level and it was unchanged after hCG-induction. This may in part be due to the Ser/Thr phosphorylation of P450c17 as demonstrated by Zhang *et al*. (1995). To confirm this notion further studies are required under *in vivo* or *in vitro* conditions. Earlier studies on meiotic maturation using hCG have often used *in vitro* conditions. To monitor the expression level of *20β-HSD* and *P450c17* precisely under *in vivo* conditions, induction with hCG in murrel is not yet successful. Since oocytes can be striped out easily in *Clarias gariepinus* following hCG-injection, *in vivo* studies were carried out to understand the precise mechanism of the steroidogenic shift.

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Abstract

20β-hydroxysteroid dehydrogenase synthesizes (*20β-HSD*) 17α, 20β-dihdryoxy-4-pregnen-3-one, the steroid required for resumption of prophase-I arrested oocytes in teleosts. The broad substrate specificity of this enzyme raises skepticism on its involvement in meiotic maturation. To study the role of *20β-HSD* in final oocyte maturation, we cloned and characterized *20β-HSD* from gonads of air-breathing catfish, *Clarias gariepinus*. Interestingly, *E. coli* expressed recombinant proteins, both full-length and an N-terminal deletion mutant catalyzed the reduction of steroids and xenobiotics, however there is significant difference between them. A positive correlation of *20β-HSD* expression was observed with both ovarian and testicular cycles. Real-time RT-PCR, Western blotting and enzyme assays have demonstrated an induction of *20β-HSD* during human chorionic gonadotropin (hCG)-induced oocyte maturation, *in vitro* and *in vivo*. Inhibition of hCG-induced oocyte maturation as well as recombinant protein catalytic activity by chemical inhibitor drugs together with immunolocalization provide new evidences for the involvement of *20β-HSD* in the final oocyte (meiotic) maturation teleosts.

Introduction

Meiotic maturation of prophase-I arrested oocytes is pre-requisite for ovulation and subsequent fertilization to occur in vertebrates (Yamashita *et al*., 2000; Nagahama and Yamashita, 2008). Studies involving several fish models revealed that oocyte maturation in teleosts are three step induction process involving gonadotropins, maturation-inducing hormone (MIH) and maturation-promoting factor (Nagahama and Yamashita, 2008). Different classes of steroid hormones have been shown to induce oocyte maturation in vertebrates including teleosts (Goswami and Sundararaj, 1974; Nagahama and Adachi, 1985; Trant and Thomas, 1989; Lutz *et al*., 2001; Senthilkumaran and Joy, 2001) apart from classical MIH, 17α, 20β-dihydroxy-4-pregnen-3-one (17α, 20β-DP; Nagahama, 1997). In general, in teleosts, progesterone derived steroids, 17α, 20β-DP and 17α, 20β, 21β-trihydroxy-4-pregnen-3-one have been identified as more potent MIHs (Nagahama and Adachi, 1985; Trant and Thomas, 1989). MIH synthesis occurs in the ovarian follicle layers under the stimulation of gonadotropins, pre-ovulatory luteinizing hormone (LH) surge being the stimulus for final oocyte maturation (FOM; Nagahama and Yamashita, 2008). A two-cell model has been proposed to explain the production of MIH (Nagahama, 1997). As per this model, the LH surge stimulates the thecal cells to produce 17α-hydroxyprogesterone (17α-OHP) that is acted up on by 20β-hydroxysteroid dehydrogenase (*20β-HSD*) in granulosa cells to produce MIH via shift in steroidogenesis (Senthilkumaran *et al*., 2004). Further, studies in some fish species demonstrated the elevation of 20β-HSD

enzyme activity in response to gonadotropins that is mimicked by forskolin and dbcAMP (Nagahama, 1997 and references therein; Kazeto *et al*., 2001). Subsequently transcriptional and translational up-regulation of *20β-HSD* were identified to be involved in the elevation of activity that is observed during FOM (Nagahama, 1997). In males, the enzymatic activity of 20β-HSD was found to be present in sperm (Sakai *et al*., 1989) and the evidence is also available for the involvement of MIH in spermiation (Miura *et al*., 1992). 20β-HSD activity was also demonstrated in non-flagellated testicular germ cells (Vizziano *et al*., 1996) indicating the importance of 20β-HSD even in early stages of gametogenesis. Further, a receptor for progesterone (17α, 20β-DP) has been identified on sperm membrane (Todo *et al*., 2000).

cDNAs encoding *20β-HSD* have been cloned from rainbow trout (Guan *et al*., 1999), ayu (Tanaka *et al*., 2002), the Nile tilapia (Senthilkumaran *et al*., 2002) and zebrafish (Wang and Ge, 2002) ovaries. Surprisingly, cDNA cloning studies have demonstrated a striking similarity of fish *20β-HSD* to that of mammalian carbonyl reductase1 (CBR1) belonging to short chain alcohol dehydrogenase/reductase (SDR) superfamily (Tanaka *et al*., 2001). Consistent with mammalian CBR1, piscine counterparts showed NADPH-dependent carbonyl reductase (CR) activity on a wide range of carbonyl compounds including several xenobiotics, endogenous prostaglandins and steroids. A relatively higher Km value for prostaglandins and steroids raises skepticism on the involvement of 20β-HSD in metabolism of steroids, *in vivo* (Wermuth, 1981; Inazu *et al*., 1992).

In light of the above cited literature that *20β-HSD* is involved in the gametogenesis and given the importance of this enzyme in different functions, to our knowledge there are no studies on the expression pattern of both transcript and protein of *20β-HSD* during FOM and spermatogenesis/testicular cycle. The cellular localization, regulation of *20β-HSD* expression and interaction with other autocrine/paracrine factors is not known in any of the lower vertebrates. Air-breathing catfish, *Clarias gariepinus* is selected as experimental model that shows annual breeding pattern characterized by an extended period of oocyte growth culminating in a single spawning event. The synchronous development of ovarian follicles in this species facilitates the correlation of the expression of steroidogenic enzyme genes with gametogenesis. Further, induced-breeding in this fish facilitate for natural collection of *in vivo* matured oocytes (from live fish) at different time-points. We used this strategy to perform both mRNA and protein expression patterns of *20β-HSD* during ovarian/testicular cycle and human chorionic gonadotropin (hCG)-induced oocyte maturation *in vitro* and *in vivo*. To enable this, we cloned and characterized *20β-HSD* from catfish gonads. Present study also utilized chemical inhibitors and immunolocalization to delineate the importance of carbonyl reductase-like *20β-HSD* in meiotic maturation. Taken together, we attempted to provide new evidences for the involvement of catfish *20β-HSD* during meiotic maturation of oocytes and also in testicular recrudescence.

Materials and Methods

Animals

Air-breathing catfish *C. gariepinus* (400-500 g) caught from local (Hyderabad, India) fresh water ponds were purchased 2-3 weeks before experimentation. Fish were acclimated for 2-3 weeks by maintaining in aquarium tanks filled with filtered tap water under normal photoperiod and ambient temperature (26± 2°C). Fish were fed with minced goat liver *ad libitum* during acclimation and experimentation. Four broadly distinguishable phases, namely preparatory, pre-spawning, spawning and post-spawning/regressed/resting were seen in both sexes of wild caught and laboratory-reared fish (Swapna *et al*., 2006). To correlate *in vitro* vs *in vivo* results, catfishes offers an excellent model system in which the induced-breeding facilitates collection of naturally matured oocytes *in vivo*. Moreover, shift in steroidogenesis in terms of plasma estradiol-17β to 17α, 20β-DP is known for catfish species (Joy *et al*., 1998; Kumar *et al*., 2000).

RT-PCR amplification of partial cDNA homologous to *20β-HSD*

Degenerate primers were designed by aligning the existing sequences of vertebrate CBR1/*20β-HSD* to clone *20β-HSD* cDNA fragment from the ovarian follicles of catfish. Using these degenerate primers (Table 1), a cDNA fragment homologous to

20β-HSD was amplified by RT-PCR and cloned in pGEM-T Easy vector (Promega, Madison, WI, USA).

cDNA library construction and screening

cDNA library was constructed from ovary and testis of catfish as per the method described earlier (Senthilkumaran *et al*., 2002). Total RNA was prepared using TRI-Reagent, (Sigma, St. Louis, MO, USA), followed by purification of poly(A)$^+$ RNA using oligotex mRNA purification kit (Qiagen, GmbH, Germany). The cDNA was synthesized from 5µg of poly(A)$^+$ RNA using ZAP cDNA synthesis kit (Stratagene, Cedar Creek, TX, USA), size fractionated by sephacryl column (Amersham, Buckinghamshire, England). The purified cDNA was ligated and packaged into UNI-ZAP-XR system using Gigapack II Gold packaging extract kit (Stratagene). Screening of the cDNA library for *20β-HSD* was performed using RT-PCR amplified cDNA fragment homologous to *20β-HSD* as probe that was radiolabeled with ^{32}P-dCTP using random primer labeling kit (Perkin Elmer, Boston, MA, USA). After three rounds of screening, positive clones were isolated after single clone excision. The resulted pBluscript phagemids were sequenced bi-directionally.

Rapid amplification of cDNA ends (RACE)

The 5' and 3' end sequence of the catfish *20β-HSD* cDNA was cloned by RNA-ligase mediated RACE system (Invitrogen, Carlsbad, CA, USA). Preparation of 5' and 3'

cDNA (RACE) templates and RT-PCR were done using gene specific primers designed (Table 1) from partial cDNA fragment as per the manufacturer's protocol and different sizes of RACE products were cloned into pGEM-T Easy vector and subsequently sequenced.

Primer	Sequence (5' – 3')	Purpose
DF1	CAGAGTGGTGAATGTVTCHAGC	Degenerate PCR
DR1	CCTGCCATGTCRGTKCKGACC	Degenerate PCR
GSP-F1	CCTGTGCAATGCCTGCTGTCCAGGA	3' RACE
GSP-F2	GGATGGGTCAGCACCGACATGGCAGA	3' RACE
GSP-R1	CACTCCGGAAGCGAGCCTGGAGGTC	5' RACE
GSP-R2	GGAACTCATTGCACAAGTCTCTGGTG	5' RACE
ORF-F	GCTAGCATGACGTCCTGTTCGG	ORF cloning
ORF-R	AGAAGCTTCCATCGCTGCACCTTCTTTTC	ORF cloning
qRT-F	GAATCGCCTTCAAAATGGCTG	Real-time PCR
qRT-R	AACATTCACCACCCTTCCTCC	Real-time PCR
β-Actin-F	ACCGAATGCCATCACAATACCAGT*	Real-time, RT-PCR
β-Actin-R	GAGCTGCGTGTTGCCCCTGAG*	Real-time, RT-PCR

Table 1. List of primers used for cloning and expression of *20β-HSD*.

* These *β-actin* primers were designed from catfish *β-actin* partial cDNA sequence and used in all subsequent chapters for gene expression studies.

Sequence analysis

Sequence analysis was done using Lasergene software (DNASTAR, Version 3). Multiple alignments were performed by ClustalW method (http//:www.ebi.ac.uk) and amino acid sequence encoded by cDNAs was deduced using Lasergene software and on line program (http//:www.expasy.org). Phylogenetic analysis was performed by neighbor-joining method using Lasergene software.

Genomic Southern analysis

Genomic DNA was prepared from ovarian follicles using genomic DNA preparation kit (Bangalore Genei, Bangalore, India), digested separately with *BamH*I, *Hind*III, *Pst*I, *Kpn*I, and *EcoR*I, electrophoresed on 0.8% agarose gel and transferred on to Hybond-N^+ nylon (Amersham) membrane by capillary transfer. Membrane was hybridized with radiolabeled cDNA of catfish ovarian *20β-HSD* and after high stringency washes signals were detected using phosphorimager (Amersham).

Northern blot analysis

Total RNAs from different stages of ovary and testis were prepared using TRI-reagent (Sigma) and 25µg of total RNA were separated on a 1% denaturing formaldehyde-agarose gel and transferred onto a nylon membrane (Hybond$^+$, Amersham). The membrane was hybridized under high stringency conditions with partial *20β-HSD* cDNA fragement (313 bp) labeled with ^{32}P-dCTP by random primer labeling kit (Perkin

Elmer). After overnight hybridization, the membrane was washed with 2XSSC, 1XSSC, 0.1XSSC containing 0.1% SDS at 60°C each for 10-15 min. The signals were detected using phosphorimager (Amersham) as well as by exposing to X-ray films with intensifying screens.

Expression of catfish recombinant *20β-HSD* cDNA in *E. coli*

Primers were designed to introduce *Nhe*I at 5' end and *Hind*III site at 3' end of catfish *20β-HSD* cDNA open reading frame (ORF). An N-terminal deletion mutant was also created through the deletion of fourteen amino acid residues using a primer with *Nhe*I site. The PCR amplified products (both full-length and N-terminal truncated) were digested with restriction enzymes, cloned in to expression vector pET28a^{+} (Novagen, La Jolla, CA, USA) and subsequently introduced into *E. coli* BL21 strain. Expression of recombinant 20β-HSD was carried out by inducing the cultures with 0.1mM IPTG at an O. D. of 0.4. One liter culture was harvested, suspended in 50mM Tris-Cl buffer pH 8.0 and homogenized by sonication. Clear lysate was obtained by centrifuging at 15,000 rpm for 30 min. Recombinant proteins were purified by Ni-NTA (Qiagen) affinity chromatography as per manufacturer's instructions.

Production of rabbit anti-catfish 20β-HSD antiserum

E. coli expressed catfish recombinant 20β-HSD protein was purified as mentioned above and further purification was carried out to remove contaminants if any, by

separating purified fractions on a 12% SDS-polyacrylamide gels followed by elution from the gels. Protein eluted from the gels was used as an antigen, mixed with Fruend's complete adjuvant and injected subcutaneously to 3 months old male rabbits. Following primary dose, booster doses were administered with Fruend's incomplete adjuvant and anti-serum was collected from marginal ear vein and anti-serum characterization was done employing Western blotting. Rabbits were housed and handled as per Institutional Animal Ethics Committee guidelines.

Enzymatic characterization of recombinant 20β-HSD

Purified recombinant proteins were incubated in 100 mM phosphate buffer pH 7.4 with the substrate [^{3}H]-17α-hydroxyprogesterone (3.44 TBq/mmol; GE healthcare, Buckinghamshire, England) in the presence of 0.1 mM NADPH at 28°C. Steroid metabolites were extracted with diethyl ether and separated on thin layer chromatography (TLC) plates using benzene:acetone (4:1 v/v) solvent system and signals were detected using phosphorimager (Amersham). The authentic 17α, 20β-DP spot that co-migrated with cold standard was extracted in ethanol and further identified by re-crystallization (Tanaka *et al*., 2002).

Determination of substrate specificity of 20β-HSD

Substrate specificity of catfish 20β-HSD was determined by the method of Wermuth (1981). Reaction mixture consisted of 100 mM phosphate buffer, pH 7.4, purified

recombinant 20β-HSD (25-100 µg), 0.08 mM NADPH, and substrates at various concentrations (water insoluble substrates were dissolved in ethanol and the final concentration of ethanol in reaction mixture did not exceed 1% (v/v) as indicated in table 2. Reactions were initiated by the addition of substrate, and decrease in absorbance at 340 nm was monitored with a Schimadzu (Japan) spectrophotometer at room temperature. Blanks without substrates and enzyme were routinely included. Inhibitors that block CR activity (phenylbutazone, indomethacin and diclofenac at 50, 10 and 100 µM concentrations respectively, were used after performing pilot studies to arrive at optimal concentrations) were added to the reaction mixtures and change in absorbance was monitored for an initial period to directly compare the enzyme activity in the presence of inhibitors.

RT-PCR analysis of tissue distribution pattern of catfish *20β-HSD*

One µg of total RNA extracted from different tissues was reverse transcribed to first-strand cDNA using Superscript III (Invitrogen) reverse transcriptase. PCR reaction was performed at 94°C for 1 min, 60°C for 30sec and 72° for 1 min for 30 cycles using a dual-block thermal cycler ABI9700 (Applied Biosystems, Foster, CA, USA).

hCG-induced oocyte maturation, *in vitro* and *in vivo*

For *in vitro* oocyte maturation studies, animals were killed by decapitation and oocytes with centrally located germinal vesicles were collected. They were then incubated in

triplicate in catfish oocyte incubation medium (Senthilkumaran and Joy, 2001) with 100 IU/ml of hCG (Pubergen, Uni-Sankyo, Hyderabad, India.). Controls were treated with saline. Follicles were collected at different time points and used for real time RT-PCR, Western blotting and enzyme activity measurement. Dead eggs were identified based on their color change and were removed with a sterile pipette. The average death rate was about 8-10%.

For *in vivo* oocyte maturation studies, fish were injected with hCG (1000 IU/kg body weight), intraperitoneally and follicles at different time points were collected by gentle stripping from ovipore and utilized for different analyses mentioned above. Both the assays were repeated thrice using different batch of female fish.

In vitro bioassay of germinal vesicle breakdown (GVBD)

In vitro bioassay of GVBD was done as described by Senthilkumaran *et al*. (2001). Fish were killed by decapitation and oocytes with centrally located germinal vesicles were selected for analysis. About 200 oocytes were then incubated at 25°C in triplicate in catfish incubation medium (Senthilkumaran and Joy, 2001) with different concentrations of hCG, 17α, 20β-DP, phenylbutazone, indomethacin and diclofenac. Phenylbutazone and diclofenac have been shown to decrease sperm-zona pellucida interaction in hamsters by inhibiting CR activity (Montfort *et al*., 2002; Concentrations of these inhibitors in GVBD assay were selected based on this report). Whenever drug treatments in combination with hCG were used, they were pretreated before the addition

of hCG as per the method of Senthilkumaran and Joy (2001). After 24 hrs, medium was removed and the oocytes were treated with Serra's fixative for checking the GVBD. The percentage of GVBD was expressed as the ratio of oocytes that undergone GVBD versus the total number of oocytes incubated.

Real-time RT-PCR

Transcript abundance of *20β-HSD* and *β-actin* (internal control) was quantified by real-time RT-PCR of total RNA isolated from the ovarian follicles collected at different durations of hCG-induced oocyte maturation, *in vitro* and *in vivo* and also at different stages of testicular and ovarian cycles. One µg of total RNA was reverse transcribed using random hexamers and MMLV-reverse transcriptase (Invitrogen) and the cDNA corresponding served as templates for each of triplicate 25 µl PCR reactions using power SYBRGreen master mix (Applied Biosystems). The PCR amplifications and fluorescence detection were performed with real-time PCR machine (ABI 7500, Applied Biosystems) under the manufacturer's universal thermal cycling conditions. *20β-HSD* and *β-actin* were amplified in a separate reaction using the same pool of cDNA template from each sample. Transcript abundance of *20β-HSD* was normalized to that of *β-actin* and *20β-HSD* mRNA relative abundance was calculated using the formula $2^{-\Delta\Delta CT}$. *20β-HSD* expression was reported as abundance relative to the values obtained for spawning phase.

Western blot analysis

A 10% (w/v) homogenate of ovarian follicles collected at different time points of hCG-induced oocyte maturation was prepared in 50mM Tris-Cl buffer pH 7.4. Protein was estimated by Bradford's (1976) method, 100µg of protein was separated on 12 % SDS-polyacrylamide gels. Subsequently, proteins from gel were transferred onto nitrocellulose membrane (Pall-Life sciences, Ann Arbor, MI, USA) and blocked with 5% (w/v) skimmed milk, then reacted with anti-cf20β-HSD polyclonal antiserum for one hour at room temperature. After washing, the membranes were incubated with horseradish peroxidase (HRP) conjugated goat anti-rabbit IgG (Bangalore Genei) or alkaline phosphatase conjugated goat anti-rabbit IgG (Bangalore Genei) secondary antibody. Following washing, blots were developed either with enhanced chemiluminisence (Amersham) or bichloropehnol indophenol and nitrobule tetrazolium (Bangalore Genei).

20β-HSD enzyme assay

Enzyme assay for estimation of 20β-HSD activity was determined by the method described in Kazeto *et al*. (2001). Homogenates of follicles collected at different time points of hCG-induced maturation, *in vitro* and *in vivo* were prepared as mentioned above and 500µg of protein was incubated for 1hr at 37°C with 3 nmol of mixture of [1,2,3 ^{3}H] 17α-OHP (10^{6} cpm) and un-labelled 17α-OHP and a co-factor generating

system (1 µmol of β-NADH, 1 µmol of β-NADP, 10 µmol glucose-6-phosphate, 2.5 IU glucose-6-phosphate dehydrogenase and 2 mmol $MgCl_2$) in 1 ml of 50 mM Tris-Cl buffer pH 7.4 containing 1 mM PMSF, 10µM leupeptin and 0.1 mM EDTA. After incubation, steroid metabolites were extracted and analyzed by TLC as mentioned above. Radioactive spots were quantified using liquid scintillation counter.

Immunocytochemisty

Immunocytochemistry was carried out using the avidin-biotin peroxidase as per method described by Swapna *et al*. (2006). In brief, sections were deparaffinized and then blocked with 10% normal goat serum for 1 hour at room temperature. The primary antibody was applied to the sections and incubated for 1 hr at room temperature in a humid chamber. Following incubation with primary antibody, sections were incubated with HRP labeled secondary antibody (Bangalore Genei) washed with PBS and developed using commercially supplied 3' 3' diaminobenzidine for 3-10 min. Images were taken with a Motic microscope fitted with Motic digital camera.

Data analysis

Data were expressed as mean ± SEM (n=3-5). Significance between groups was tested by ANOVA followed Student's-Newman-Keuls' test using Sigmastat3.1 software. Differences among groups were considered significant at $p<0.05$.

Results

Molecular cloning of *20β-HSD* from gonads of catfish

A 313 bp partial cDNA fragment homologous to *20β-HSD* was obtained from catfish ovary and testis by RT-PCR (Fig. 1). Approximately 7.5 x 10^5 recombinant phages of catfish testis library were screened with a 313 bp *20β-HSD* partial cDNA probe. After three rounds of screening, four positive clones were obtained and they were sequenced from both the ends. The testicular *20β-HSD* was 1.195 kb long with a 754 bp 3' untranslated region (UTR) having polyadenylation signal (Fig. 2). The open reading frame encoded a protein of 146 amino acids with TyrXXXLys conserved catalytic site of the SDR superfamily. However, all the clones were truncated at 5' end. The missing region in the truncated clones contained the conserved Rosmann fold (GlyXXXGlyXGly). Having obtained the truncated clones by screening the testicular library, full-length cDNA of catfish *20β-HSD* was obtained from ovarian follicles by 5' and 3' RACE using gene specific primers designed from partial cDNA fragment (Fig. 1). Catfish ovarian *20β-HSD* was 1.178 kb in length with 60 bp 5' UTR and 246 bp 3' UTR. The 3' UTR contained a polyadenylation signal (Fig. 3). The protein encoded by the ovarian cDNA has 290 amino acids, 14 amino acids longer at N-terminal than the other vertebrate counterparts (Fig. 4).

Phylogenetic analysis showed that the catfish ovarian *20β-HSD* shares highest homology with that of zebrafish ovarian CR/*20β-HSD* and segregated into a separate

clade (Fig. 5). The signature domains typical of SDR superfamily including Rossamann fold and the catalytic domains were found in the catfish ovarian *20β-HSD* and ClustalW multiple alignment analysis demonstrated that these regions are highly conserved among all the vertebrates (Fig. 4).

Genomic Southern analysis

Genomic Southern analysis identified two bands with *Kpn*I, *Hind*III and *Pst*I while it was a single band with *BamH*I when probed with ORF region of *20β-HSD* (Fig. 7).

Northern blot analysis

Northern blot analysis was carried out to find out the transcript sizes in gonads. Northern blot analysis identified a single transcript of about 1.1 kb in ovary while two transcripts of 1.1 and 1.6 kb were detected in testis (Fig. 8).

Characterization of 20β-HSD enzyme activity of the recombinant cDNA product

Catfish 20β-HSD has an extra N-terminal 14 amino acid sequence, we intended to analyze whether it has any role in enzymatic activity. Taking the advantage of extra N-terminal sequence, a deletion mutant was constructed. Both the ORFs (Normal ORF is designated as ORF1 and deletion mutant is designated as ORF2) were cloned into pET28a and sequences were verified. Proteins expressed and purified (Fig. 9A) from both ORFs were confirmed by Western blot with anti-His antibody (Amersham; Fig.

9B). The purified proteins were incubated with 17α-OHP in presence of NADPH and resulting steroid metabolites were separated by TLC (Fig. 10). A band that co-migrated with authentic 17α, 20β-DP was extracted and re-crystallized and the extraction ratio from three crystallizations in different solvent systems was almost same demonstrating that the *20β-HSD* cDNA clones indeed possess 20β-HSD activities. The efficiency of conversion of 17α-OHP was higher for ORF2 than ORF1.

Substrate specificity and CR activity of 20β-HSD

The recombinant proteins also reduced several carbonyl compounds like other vertebrates CRs (Table 2A). Like other CRs, catfish 20β-HSD efficiently catalyzed the reduction of quinones (menadione) whereas steroids were reduced at lower rates. There was a significant difference in CR activity between the two ORF constructs with the N-terminal deletion construct exhibiting higher activity. Catfish 20β-HSD was inhibited by diclofenac, phenylbutazone and indomethacin (Table 2B).

Spatial expression pattern and immunolocalization in gonads

Semi-quantitative RT-PCR analysis of tissue distribution pattern of catfish *20β-HSD* demonstrated that the transcript is ubiquitously distributed in all the tissues tested with abundant expression in gonads, brain, gill, kidney and a low level in liver (Fig. 11A). Western blot analysis of 20β-HSD protein expression in different tissues was in

accordance with *20β-HSD* transcript (Fig. 11B). Immunocytochemical analysis demonstrated 20β-HSD immunoreactivity in the follicular layer of the ovary while in testis immunoreactivity was observed in interstitial cells, spermatogonia and spermatocytes (Fig. 12). Anti-serum characterization details were presented in figure 13(A, B, & C).

Changes in mRNA and protein levels during gonadal cycle

To investigate the stage dependent expression of catfish *20β-HSD*, we analyzed transcript level by real-time quantitative RT-PCR from total RNA prepared at different stages of ovarian cycle. Expression analysis of *20β-HSD* during ovarian cycle revealed an increase from preparatory phase to spawning phase followed by a decrease in regressed phase (Fig. 14). Western blot analysis of 20β-HSD protein levels during different stages of ovarian cycle was in correlation with transcript levels (Fig. 14). *20β-HSD* transcript and protein levels during testicular cycle also showed a positive correlation (Fig. 14).

Oocyte maturation *in vitro* and *in vivo*

Expression of catfish *20β-HSD* during hCG-induced oocyte maturation was assessed by real-time quantitative RT-PCR and Western blot methods. In *in vitro* oocyte maturation, *20β-HSD* mRNA level augmented from 0 hrs to 2 hrs after induction with hCG, reached

its peak level by 4 hours and then a decrease was noticed by 8 hrs. In hCG-induced oocyte maturation *in vivo*, *20β-HSD* transcript level increased steadily from 4hrs and achieved maximum level at 12 hrs (Fig. 15). Both in *in vitro* and *in vivo* conditions, 20β-HSD enzyme activity as well as protein level followed a similar trend like mRNA level (Figs. 17 & 18).

Effect of CR inhibitors on *in vitro* oocyte maturation

Oocytes underwent GVBD with 17α, 20β-DP (Fig. 19A) by 8-10 hrs where as GVBD was observed by 20-24 hrs when incubated with hCG (Fig. 19B) and this was used as standard to compare effect of phenylbutazone, indomethacin and diclofenac on hCG-induced oocyte maturation. All the drugs inhibited hCG-induced oocyte maturation significantly in a dose-dependent manner (Fig. 20).

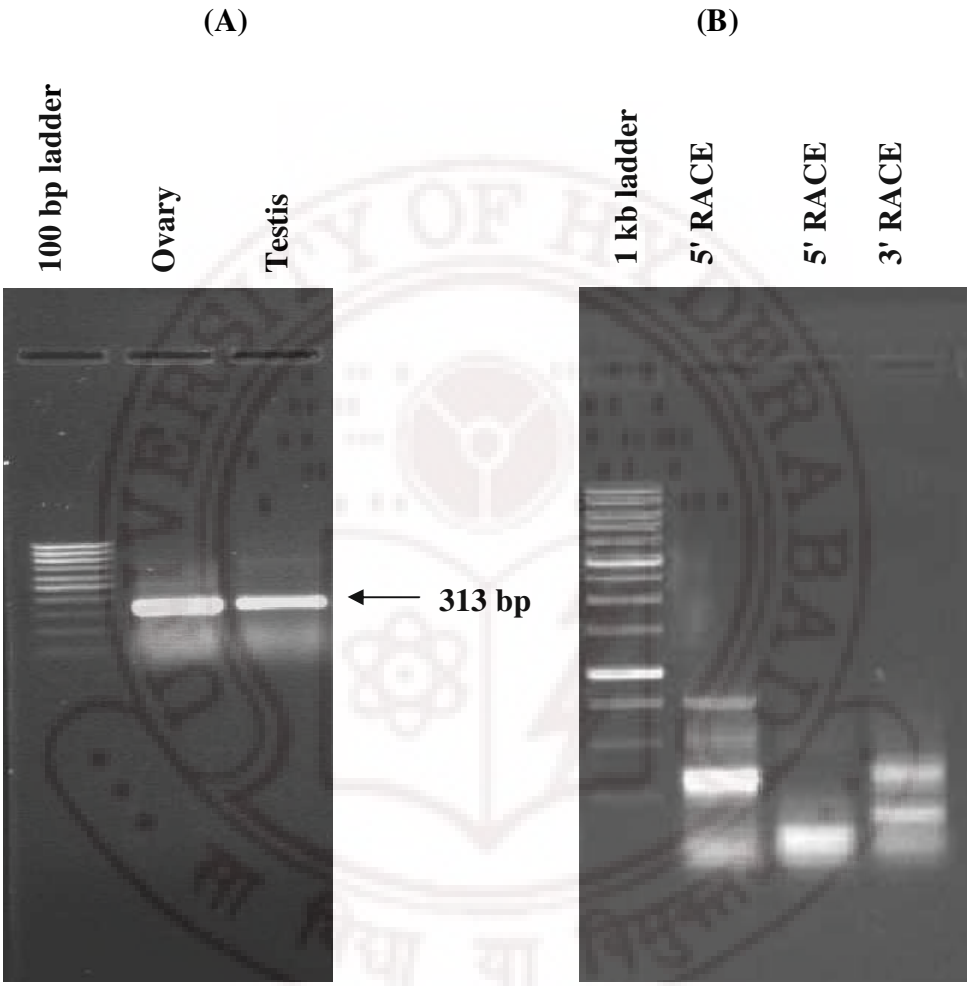

Fig. 1. 1% Agarose gels showing RT-PCR products using degenerate primers for amplification of partial *20β-HSD* cDNA fragment (A), 5' and 3' RACE (B) products using gene specific primers to obtain *20β-HSD* full-length cDNA.

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                                        gagtacggcggcgtggacgtgctcgtg 27
                                         E  Y  G  G  V  D  V  L  V
aacaacgcgggaatcgccttcaaaatggctgataaaacacctttcgggatccaggctgat 87
 N  N  A  G  I  A  F  K  M  A  D  K  T  P  F  G  I  Q  A  D
gtgacgctcaagactaacttctttgccaccagggacttgtgcaatgagttcctccccatc 147
 V  T  L  K  T  N  F  F  A  T  R  D  L  C  N  E  F  L  P  I
atcaagccgggaggaagagtggtgaatgtatctagcgtcatgggttccatcgccctgagc 307
 I  K  P  G  G  R  V  V  N  V  S  S  V  M  G  S  I  A  L  S
cgctgcagcccggacctccaggctcgcttccggagtgatgacatcaccgaggaggaactg 367
 R  C  S  P  D  L  Q  A  R  F  R  S  D  D  I  T  E  E  E  L
gtggggctaatggagcgcttcgtggcggatgctaaagaggaagctcacactcaaaggggc 427
 V  G  L  M  E  R  F  V  A  D  A  K  E  E  A  H  T  Q  R  G
tggcccgacaccgcatatggcatctccaagactggcttgaccaccctgaccaggatccat 487
 W  P  D  T  A  Y  G  I  S  K  T  G  L  T  T  L  T  R  I  H
gcccggaaactgacgcaggagagacctggtgatgaaatcctgtgcaatgcctgctgtcca 547
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aaaaaaaaaaaa                                                1333
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Fig. 2. Partial nucleotide (blue) and deduced amino acid (red) sequence of catfish testicular *20β-HSD*. UTRs are shown in black letters and polyadenylation signals are shown in boldface letters with underline.

Fig. 3. Nucleotide (blue) and deduced amino acid (red) sequence of catfish ovarian *20β-HSD*. UTRs are shown in black letters and polyadenylation signal is shown in boldface letters underlined.

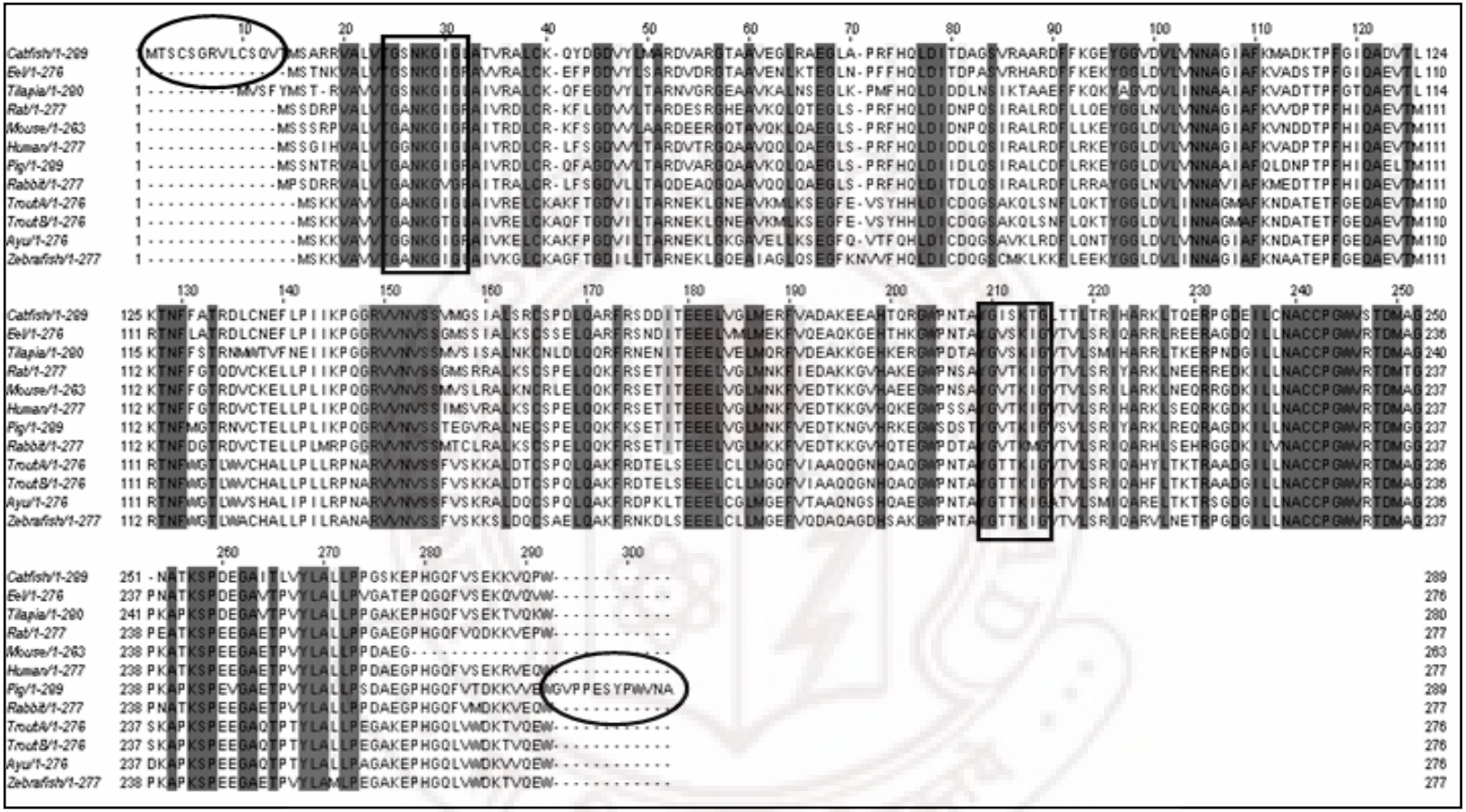

Fig. 4. ClustalW multiple alignment of catfish *20β-HSD* with other vertebrate counterparts (Accession No.: Human **NM001757**; Mouse **NM007620**; Rat **NM019170**; Pig **M80709**; Rabbit **NM001082749**; Tilapia **AF439713**; Trout A **NM001124596**; Trout B **NM001124255**; Eel **AF420278**; Ayu **D82967**; Zebrafish **AF298898**). Highly conserved regions are shaded. The conserved domains are shown in rectangle. A extra N-terminal sequence in catfish *20β-hsd* and a extra C-terminal sequence in pig testicular 20β-HSD are shown in circle.

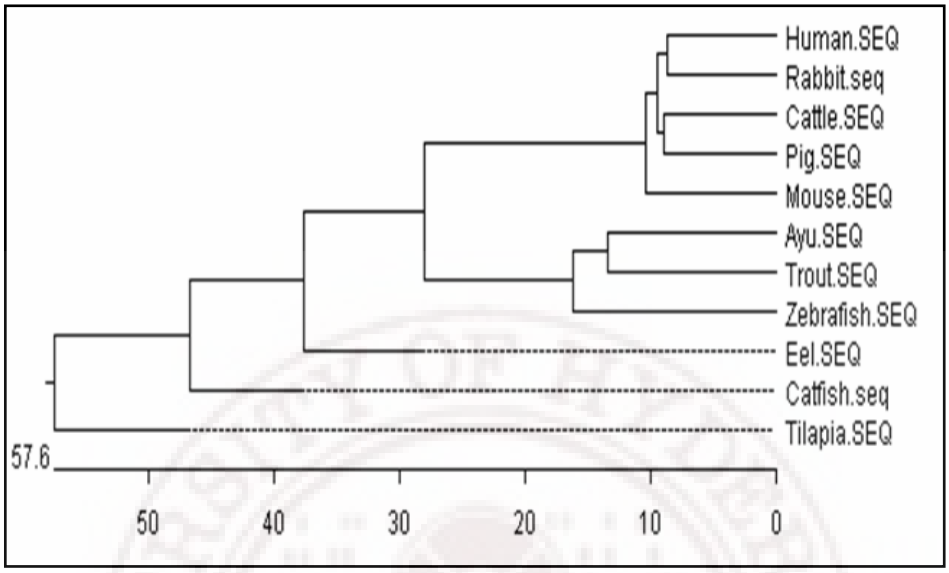

Fig. 5. Phylogenetic tree showing the evolutionary status of catfish *20β-HSD* (Please refer Fig. 4 for accession numbers).

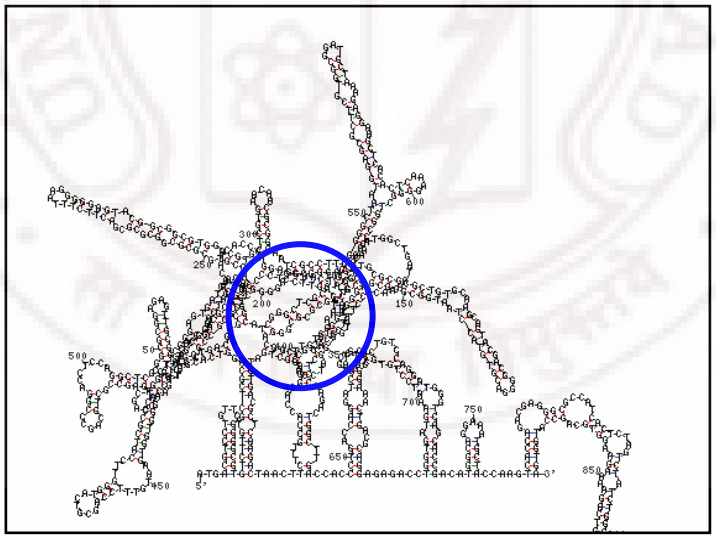

Fig. 6. *20β-HSD* mRNA secondary structure determined by mfold analysis. region shown in circle has very high complexity with greater than 75% GC content, high Tm.

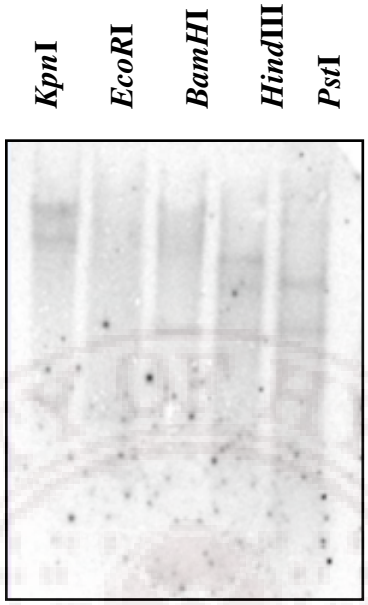

Fig. 7. Southern blot analysis of genomic DNA extracted from catfish ovary, digested with different restriction endonucleases and probed with ORF region of the *20β-HSD* cDNA clone.

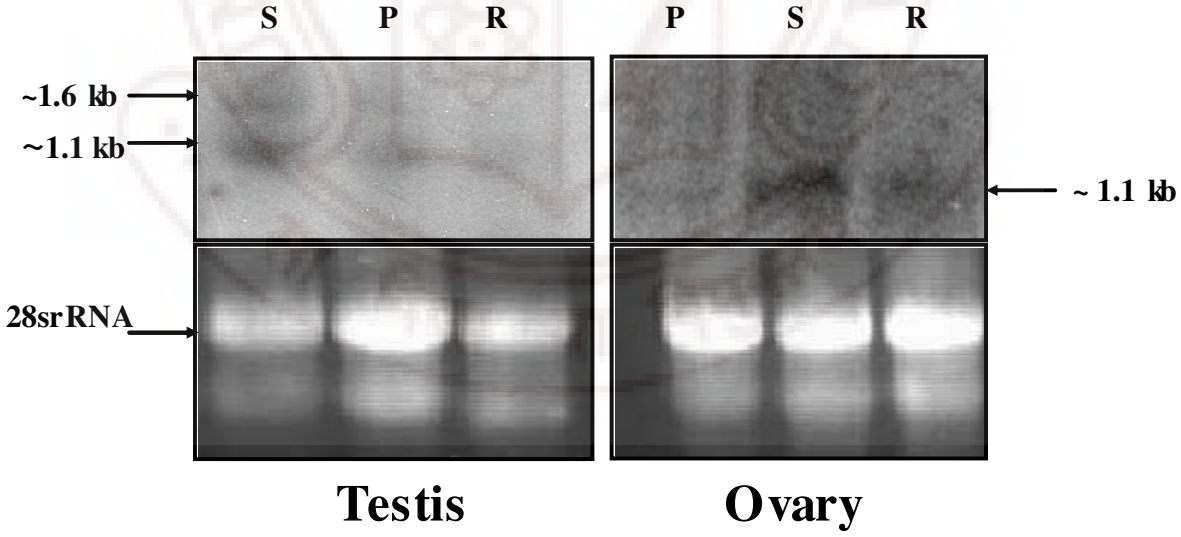

Fig. 8. Northern blot analysis of total RNA (25µg) from different stages of ovary and testis. Lower panel shows the ethidium bromide staining. P, preparatory; S, spawning; R, regressed.

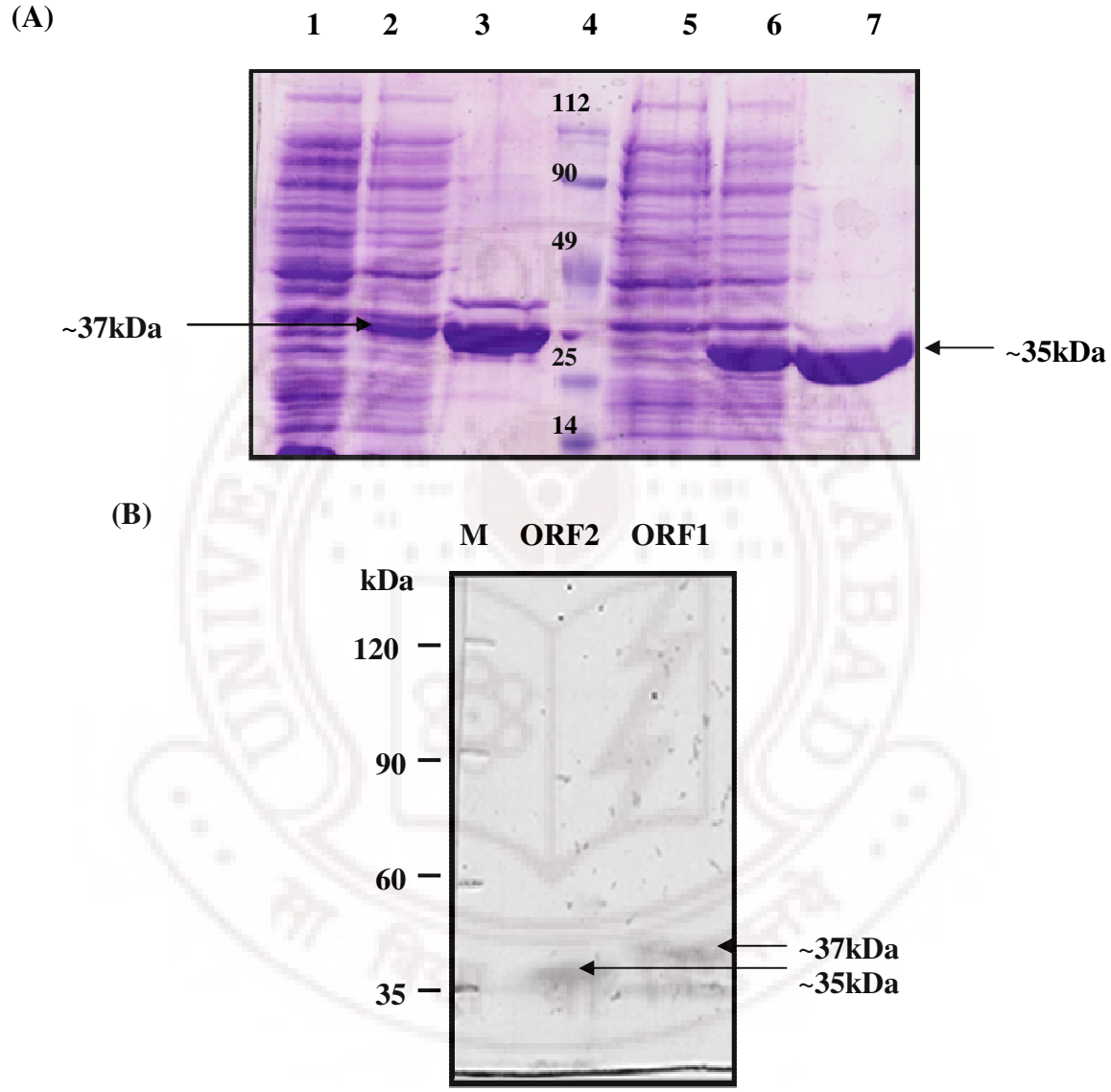

Fig. 9. 12% SDS-PAGE showing catfish 20β-HSD over-expressed in *E. coli* (A). Lanes 1 ORF1 un-induced, 2 ORF1 induced, 3 ORF1 purified, 4 Molecular weight marker (sizes in kDa), 5 ORF2 un-induced, 6 ORF2 induced and 7 ORF2 purified. Western blot analysis with anti-His antibody confirming the catfish recombinant 20β-HSD (B).

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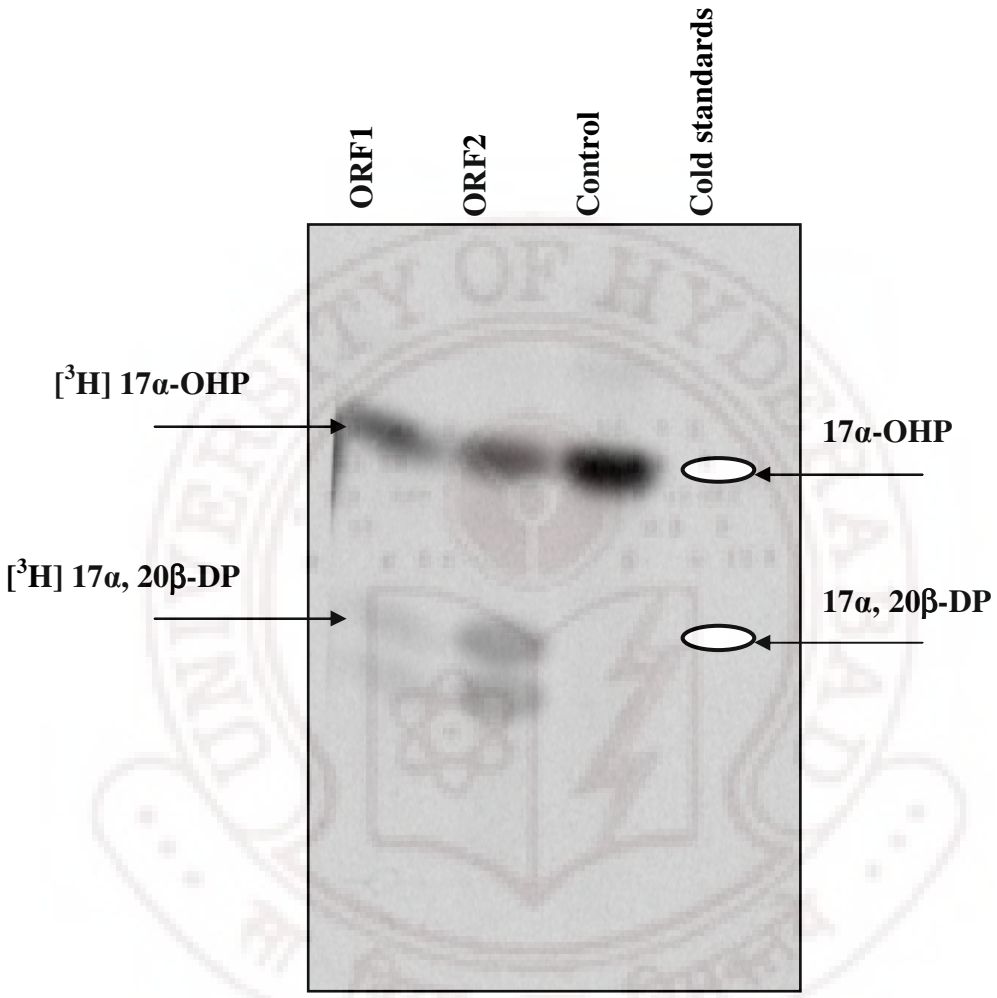


Fig. 10. Autoradiogram showing functional analysis of recombinant 20β-HSDs. Purified recombinant 20β-HSD was incubated with 17α-OHP, reaction metabolites were extracted and separated by TLC. Arrowheads at right side indicate location of 17α-OHP and authentic 17α, 20β-DP and left side cold standards.

(A)

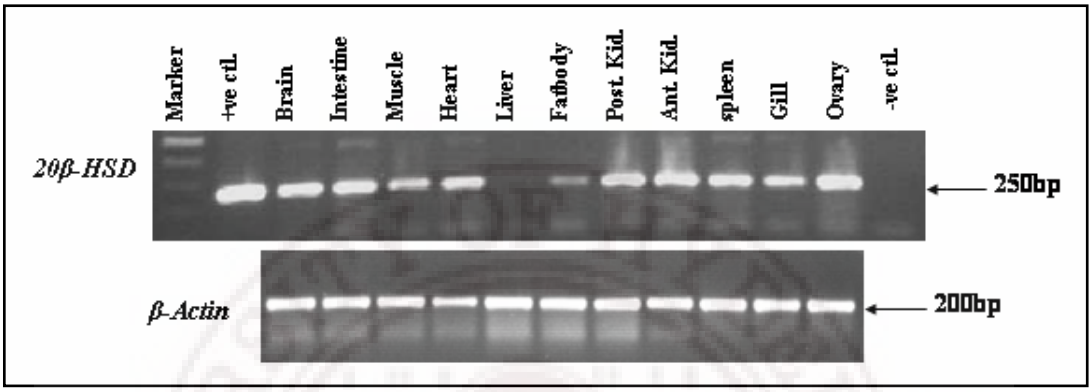
Substrate	Concent-ration (mM)	Specific activity (pmol/mg-protein/min)		Relative Velocity (%)	
		ORF1	ORF2	ORF1	ORF2
4-Nitrobenzaldehyde	0.5	260	598	100	230
Menadione	0.25	118	418	45	160
17α-OHP	0.02	51	52	19.6	20
Progesterone	0.02	41	48	15.7	18.4
Pregnenolone	0.02	32	36	12.3	13.8
Androstenedione	0.05	25	28	9.6	10.8
Testosterone	0.05	38	36	14.6	13.8
Dehydroepiandrosterone	0.05	64	45	24	17.3

(B)

Inhibitor	Concentration (μM)	% Inhibition
Phenylbutazone	50	51
Diclofenac	100	66
Indomethacin	10	69

Table 2. CR activity of catfish recombinant 20β-HSD with xenobiotics and steroids (A). Relative velocity is expressed as percent activity with that of 4-nitrobenzaldehyde set at 100. Inhibition of catfish 20β-HSD by carbonyl reductase inhibitors (B). Inhibition reactions were carried out with 0.5 mM menadione as substrate, purified ORF1 was used for inhibitions (refer text for further details).

(A)



(B)

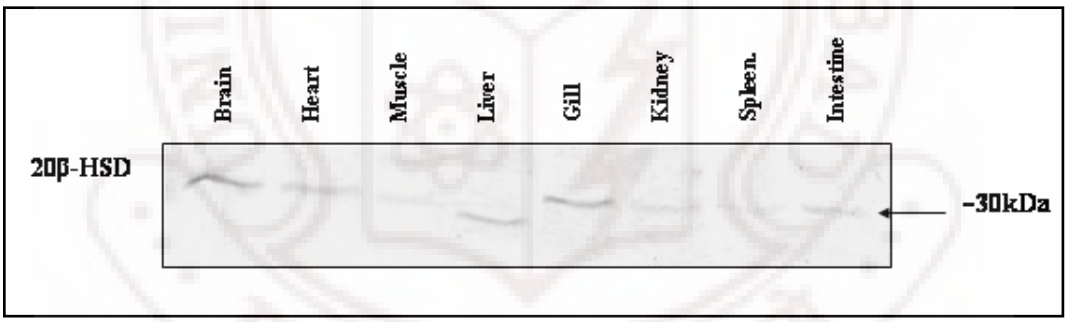


Fig. 11. Spatial expression pattern of *20β-HSD* mRNA in the catfish tissues (A; Upper panel) and β-actin (lower panel). The plasmid clone of *20β-HSD* cDNA was used as positive control (+ve ctl) and negative control (-ve ctl) contains no cDNA template. Post. Kid., Posterior kidney; Ant. Kid, Anterior kidney. Western blot analysis demonstrating the 20β-HSD protein expression pattern in various tissues of catfish(B).

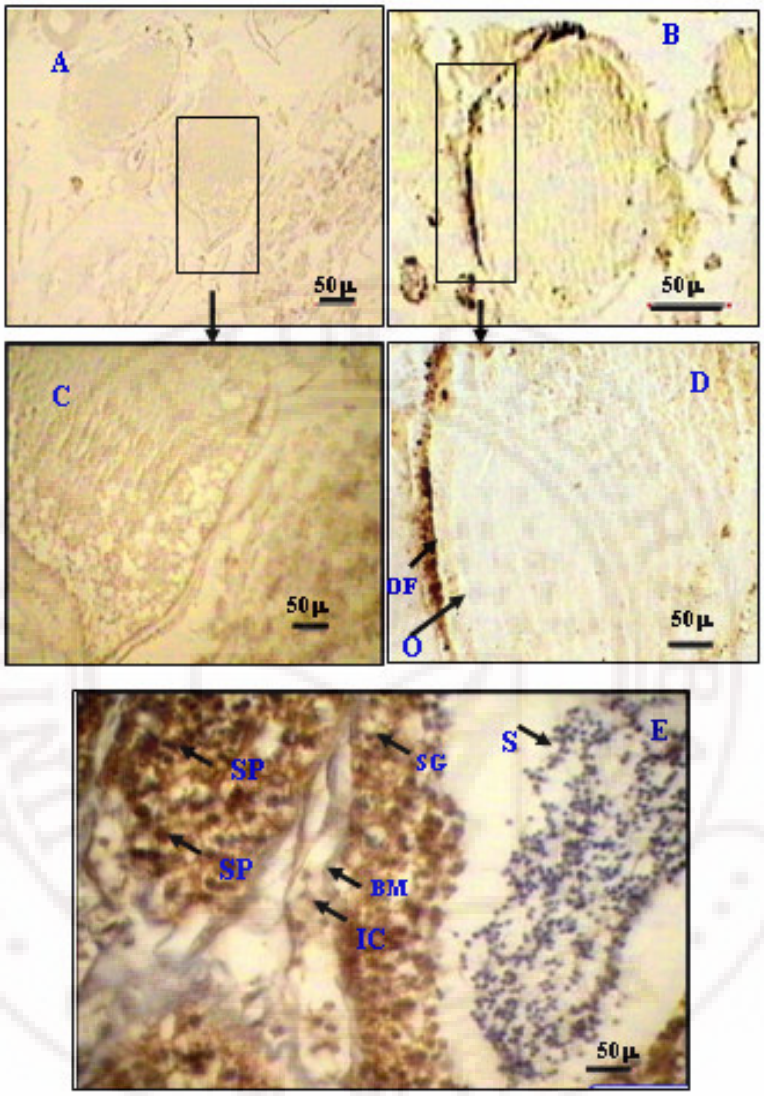


Fig. 12. Immunocytochemical localization of 20β-HSD in catfish gonads. Regions of follicular layer showed immunoreactivity in ovary (B). Pre-immune serum was used as –ve control (A). C & D represents higher magnification pictures corresponding to A & B. Interstitial cells (IC), spermatogonia (SG) and spermatocytes showed immunoreactivity in testis (E). O, oocyte; OF, ovarian follicular layer; BM, basement membrane; S, spermatids.

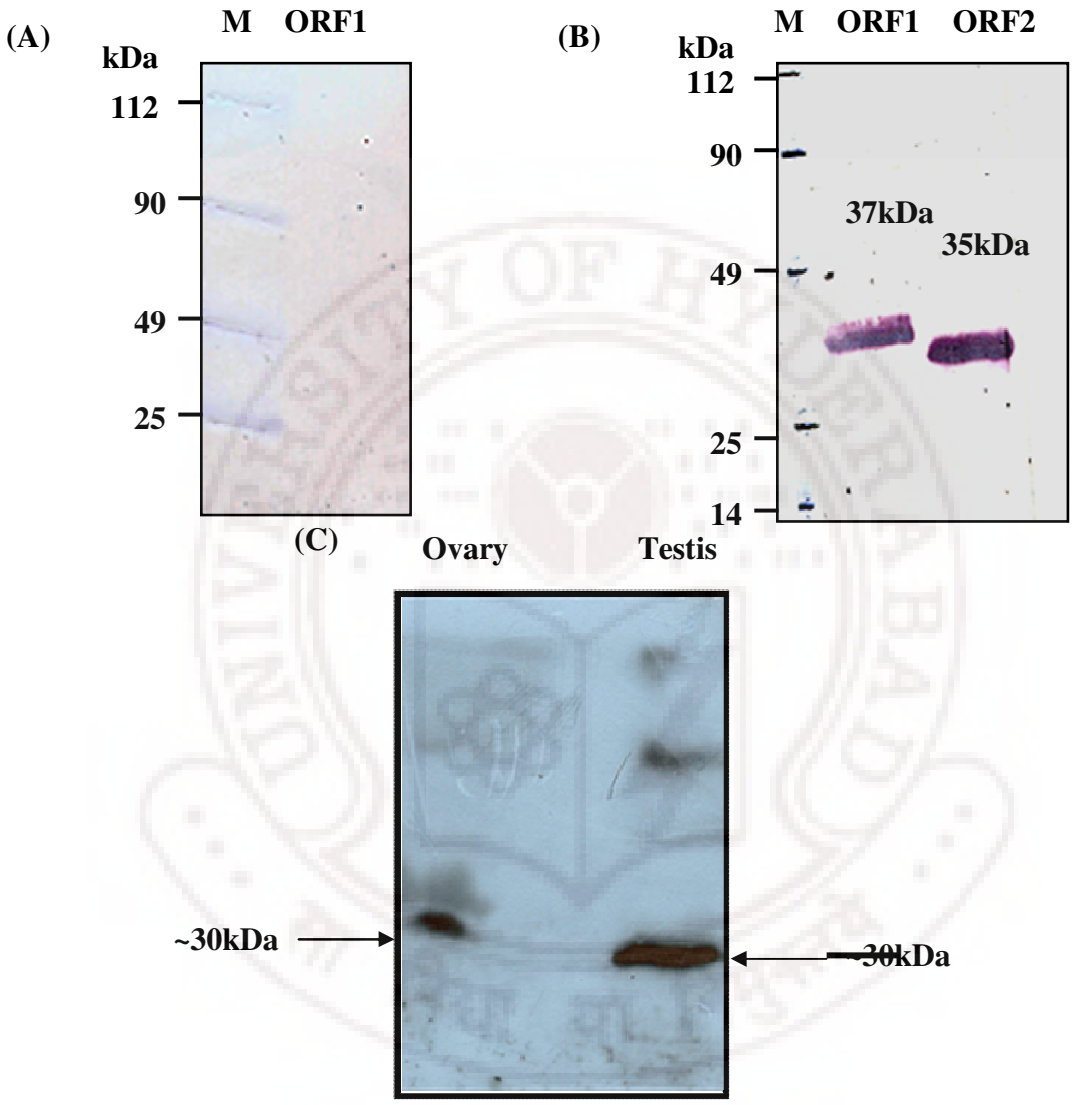


Fig. 13. Western blot analysis demonstrating the antibody characteristics of rabbit anti-cf20β-HSD. Purified catfish recombinant 20β-HSD detected with pre-immune serum (A). Detection of purified recombinant catfish 20β-HSD protein by anti-cf20β-HSD (B). Detection of 20β-HSD in catfish ovary and testis with anti-cf20β-HSD (C).

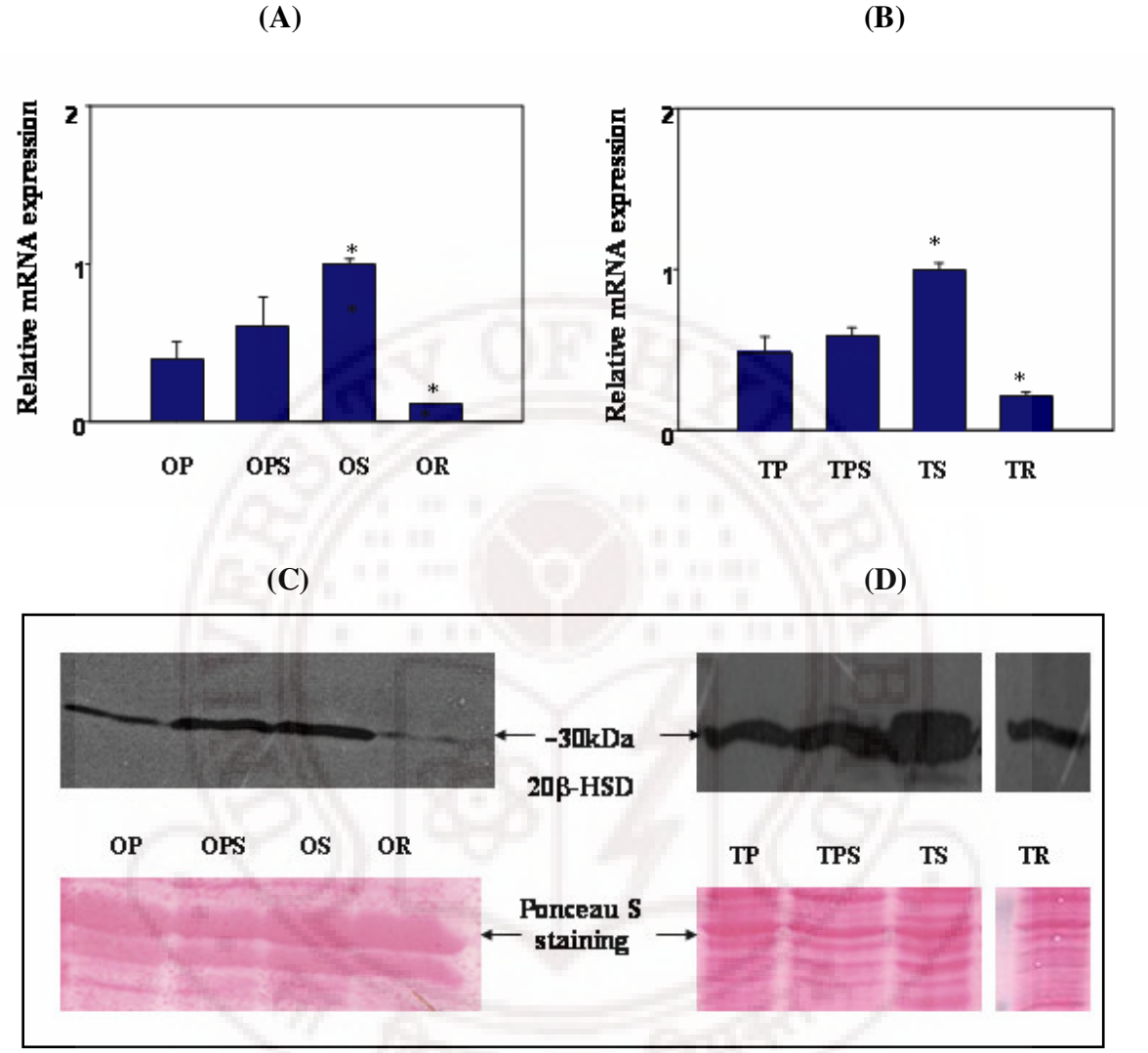


Fig. 14. Stage dependent expression of *20β-HSD* in catfish ovary (A) and testis (B) by real-time RT-PCR. *20β-HSD* expression is reported as fold change relative to spawning phase. * indicates significance (n=3, ANOVA, *P* <0.05). Western blot analysis showing 20β-HSD expression (Upper panel) in different stages of ovarian (C) and testicular (D) cycles. Data is representative of three independent experiments. Ponceau S staining (lower panel) was used to depict equal loading. OP, ovary preparatory; OPS, ovary pre-spawning; OS, ovary spawning; OR, ovary regressed; TP, testis preparatory; TPS, testis pre-spawning; TS, testis spawning; TR, testis regressed.

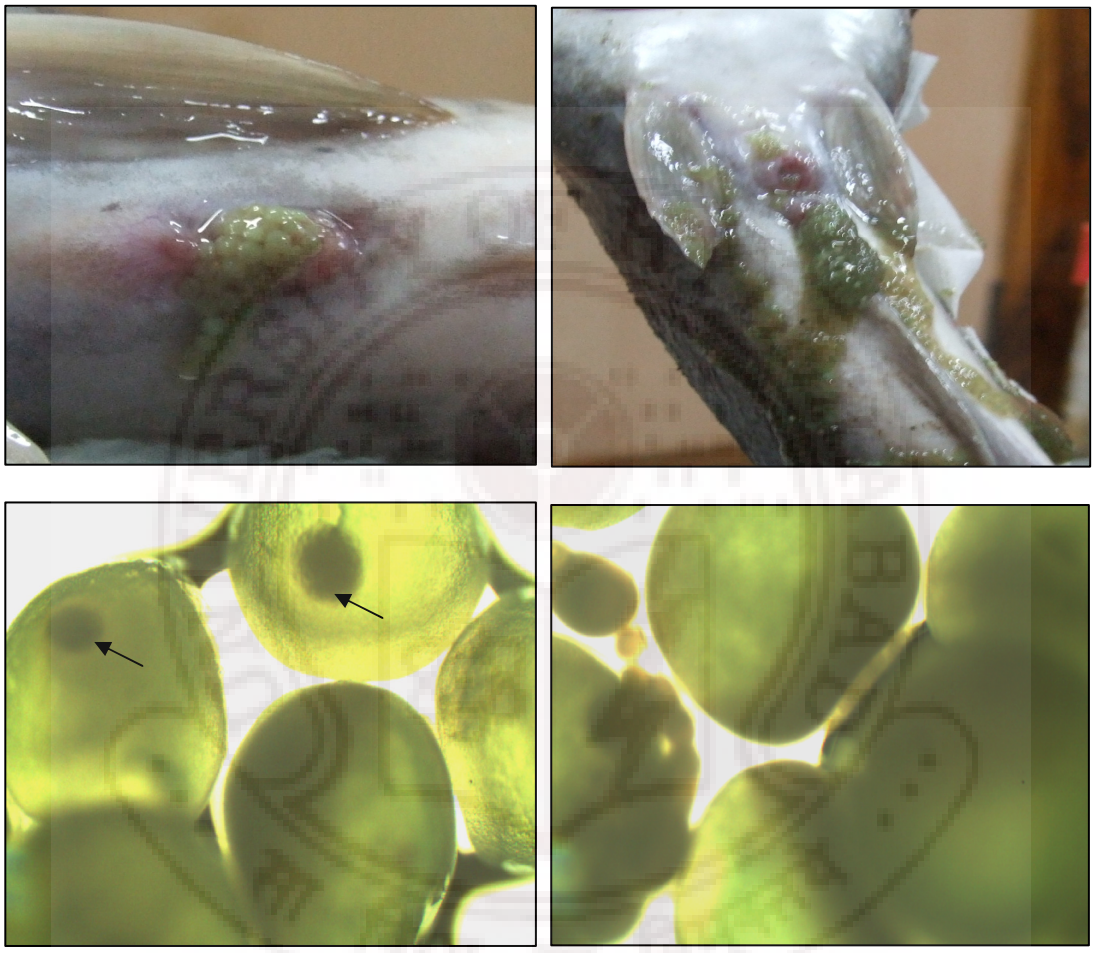


Fig. 15. Photographs (upper panel) showing the oocytes collection before and after treatment with hCG. Photomicrographs (lower panel) showing the germinal vesicle (GV; arrows) before treatment with hCG. GV was not seen in oocytes after treatment with hCG.

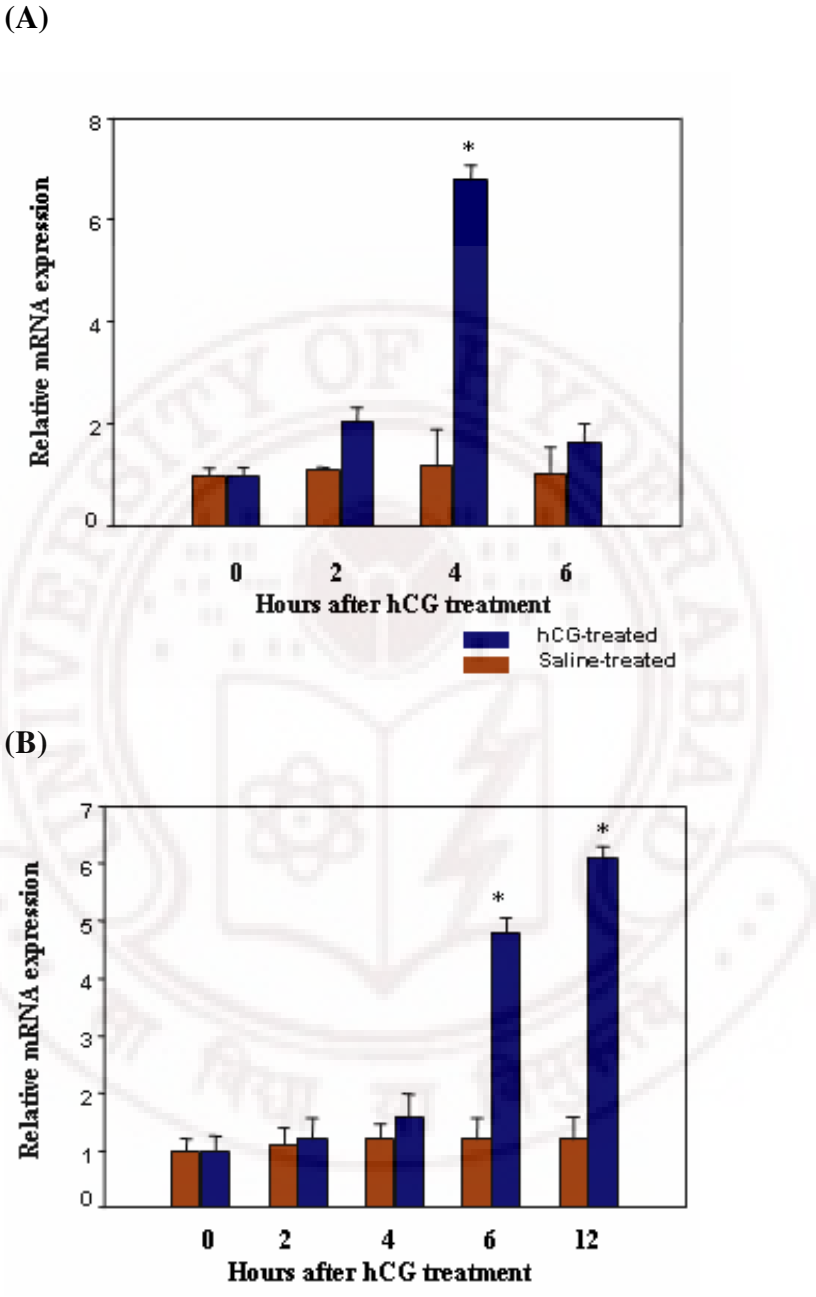


Fig. 16. Real-time RT-PCR analysis demonstrating the expression of *20β-HSD* during hCG-induced oocyte maturation in vitro (A) and in vivo (B). * Indicates statistical significance, n=3, ANOVA).

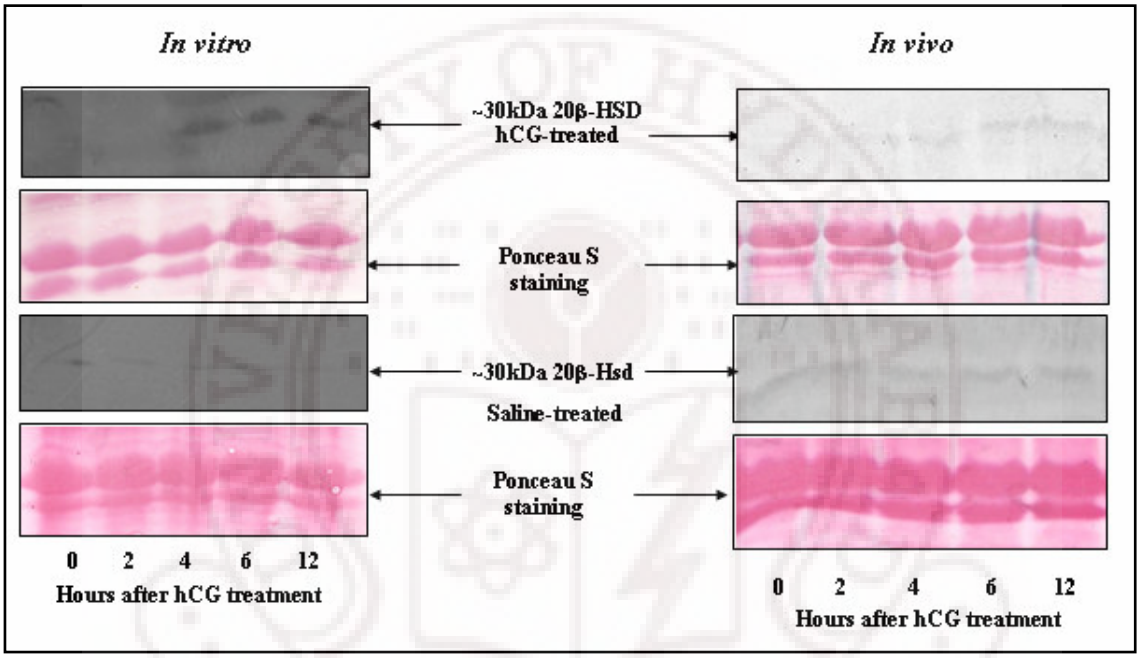
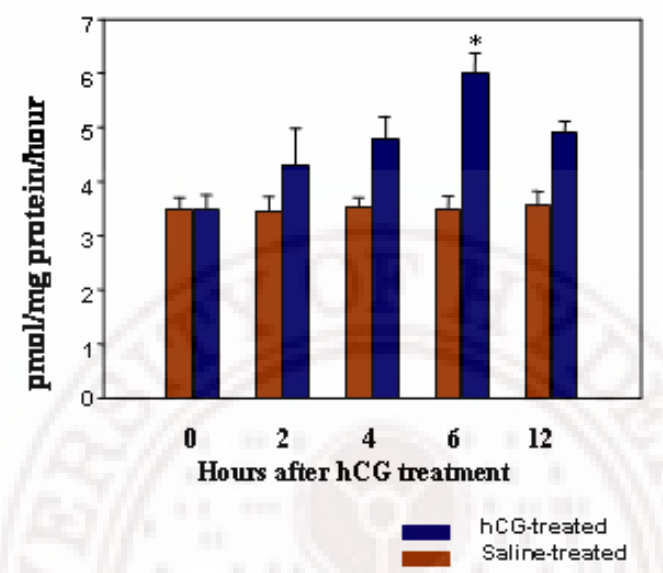


Fig. 17. 20β-HSD protein level during hCG-induced oocyte maturation assessed by Western blotting (representative, n=3).

(A)



(B)

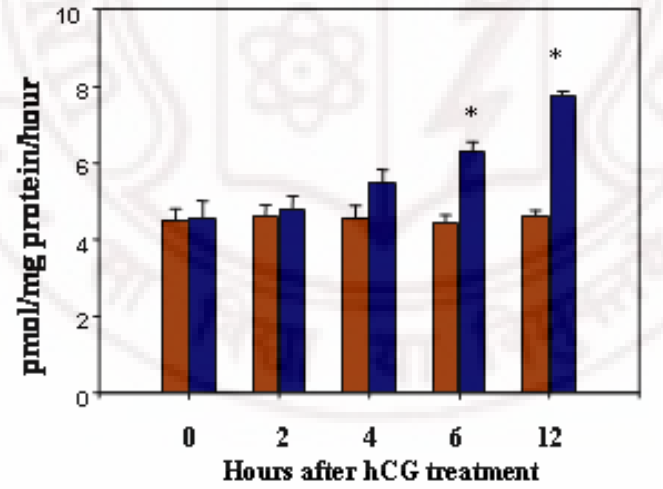


Fig. 18. 20β-HSD activity during hCG-induced oocyte maturation, *in vitro* (A) and *in vivo* (B) (* indicates significance, n=3, ANOVA, *P*<0.05).

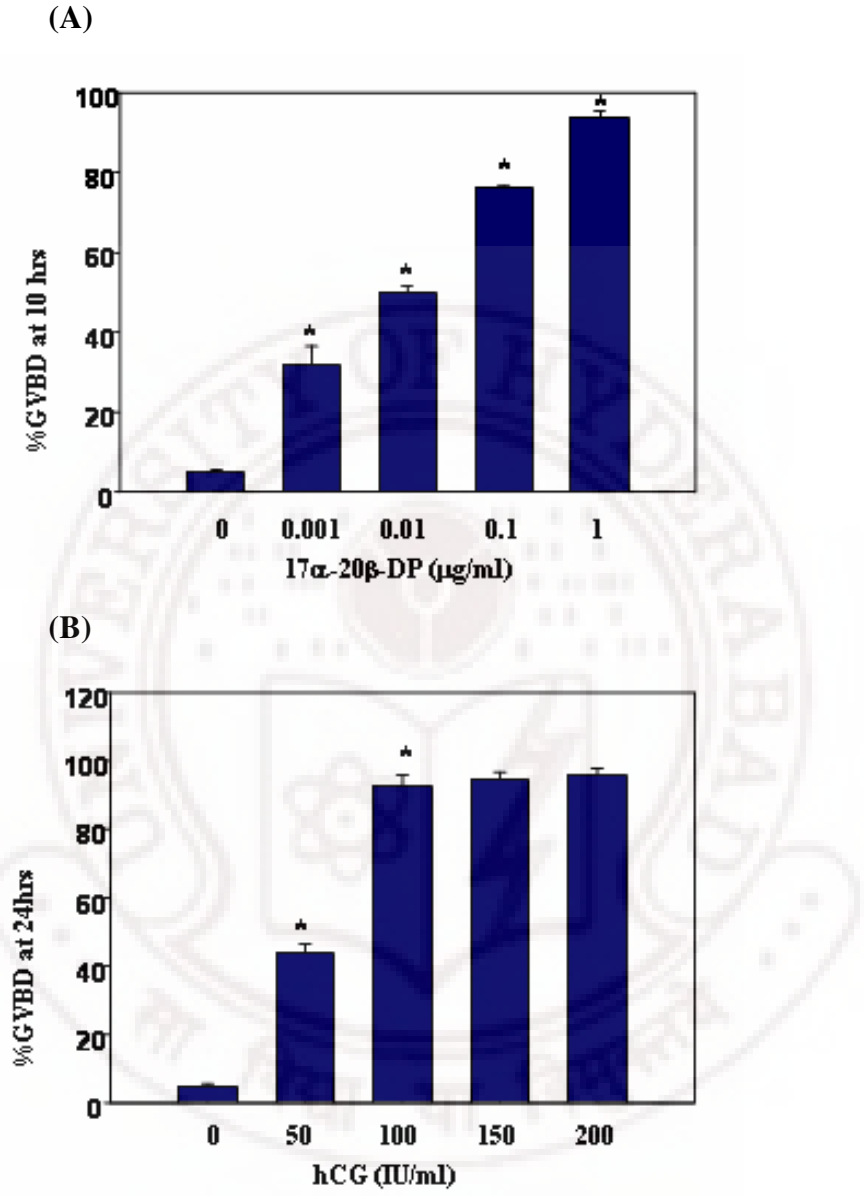


Fig. 19. Percent GVBD induced by different doses of 17α, 20β-DP (A) and hCG (B). * Indicates significance, n=5, ANOVA, *P*<0.05).

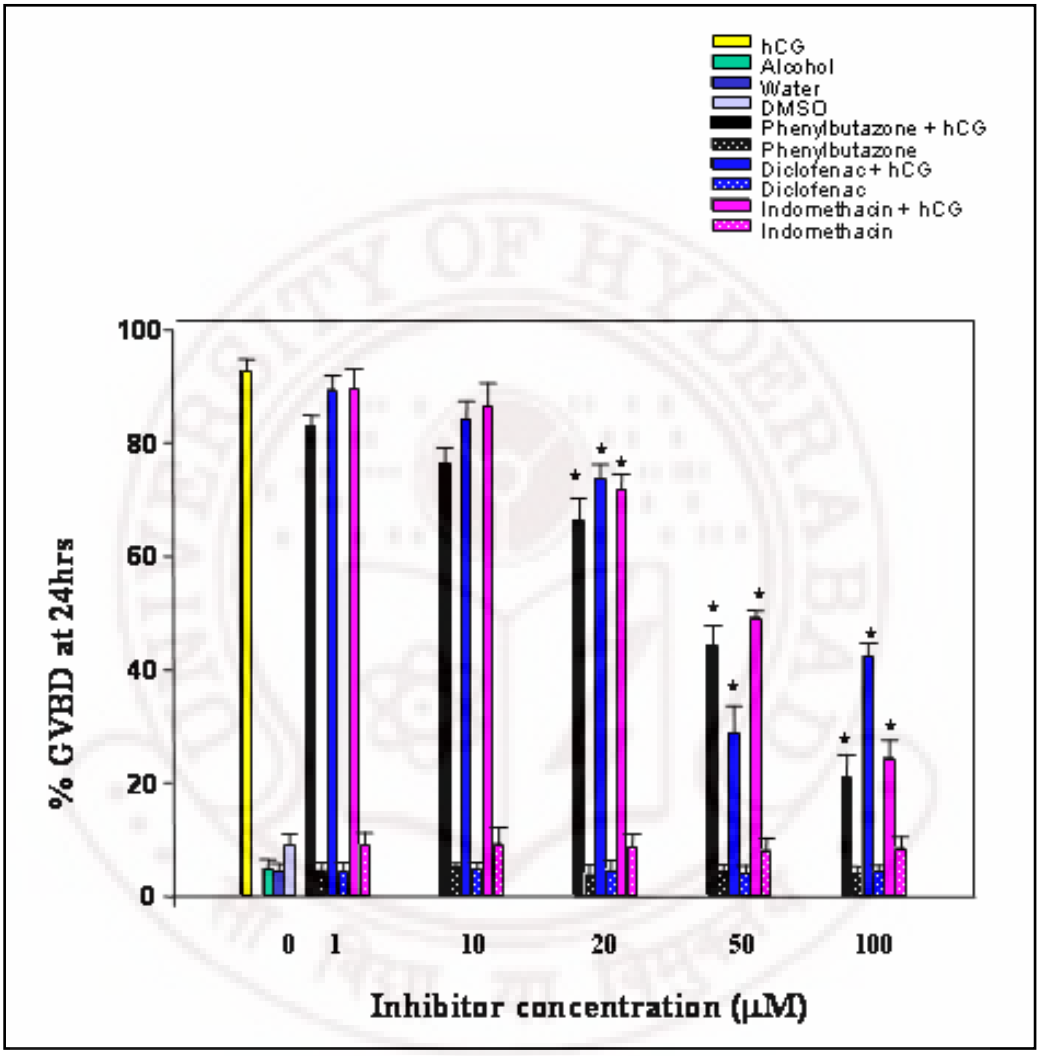


Fig. 20. Percent GVBD induced by different doses of CR inhibitors phenylbutazone, diclofenac and indomethacin in the presence of hCG (* indicates significance, n=3, ANOVA, *P*< 0.05).

Discussion

Primary goal of the present study is to confirm the role of *20β-HSD* in oocyte maturation under *in vitro* and *in vivo* conditions. None of the earlier reports provided substantial evidences using enzyme activity, chemical inhibitor drugs and immunolocalization studies as done in the present work to implicate *20β-HSD* in FOM. *20β-HSD* cDNAs from ovary and testis are 3' UTR variants, testicular one being longer. However, genomic Southern analysis tends to indicate a single copy gene for *20β-HSD* in catfish. Our report was in accordance with tilapia, where single copy of *20β-HSD* gene was denoted (Senthilkumaran *et al*., 2002). But the presence of multiple forms of *20β-HSD*/CR is not uncommon and has been found in the ovaries of rainbow trout (Guan *et al*., 2002), rat (Aoki *et al*., 1997) and hamster (Tereda *et al*., 2001). Studies pertaining to structure function relationship utilizing site-directed mutagenesis and X-ray crystallography have demonstrated that the co-factor binding domain Rossmann fold and active site motif are crucial (Daux and Gosh, 1997; Guan *et al*., 2000). The SDR super-family is one of the biggest families with more than 2000 known primary structures (Kallberg *et al*., 2002). In spite of highly divergent primary structures of this family, they all have super-imposable tertiary structures; highly conserved signature domains and these motifs are well conserved in catfish *20β-HSD* cDNAs too.

Purified recombinant protein from *E. coli* expressing *20β-HSD* cDNAs of catfish (Both full ORF and N-terminal deletion mutant) catalyzed the conversion of 17α-OHP

although ORF2 was efficient than ORF1. Like catfish 20β-HSD, pig testicular 20β-HSD has an extra sequence but at C-terminus (Tanaka *et al*., 1991) and has shown to influence the substrate specificity (Itoda *et al*., 2002). The observed low enzyme activity of ORF1 might be due to the presence of extra sequence at N-terminal that would pose a limitation for the availability of co-factor. However, much detailed studies are necessary to confirm this notion. Besides their role in reproduction, in mammals 20β-HSDs/CBR1 have been implicated in the reduction of several xenobiotic compounds. Consistent with mammalian CBR1, *E. coli* expressed 20β-HSD of fishes, including catfish reduced xenobiotic compounds.

Analysis of spatial expression pattern of catfish *20β-HSD* by semi-quantitative RT-PCR demonstrated ubiquitous expression in all the tissues tested with an abundant expression in ovary, testis, brain, gill and kidney. The abundant expression of *20β-HSD* in gill and kidney is in accordance with previous reports (Guan *et al*., 1999; Senthilkumaran *et al*., 2002; Tanaka *et al*., 2002; Wang and Ge, 2002). Gills are directly exposed to the environment and the kidneys in teleosts continuously produce large amounts of urine containing 17α, 20β-DP (Virmeirssen and Scott, 1996). The high level of *20β-HSD* in these organs supports the notion that these are responsible for the local conversion of 17α-OHP to 17α, 20β-DP and is evidenced by the identification of 20β-HSD activity in gills of cyprinid and salmonid fishes (Kime and Ebrahimi, 1997). During spawning period, large amount of 17α, 20β-DP released into the environment could serve as

pheromone (Stacey *et al*., 1989). Though role of *20β-HSD* has been firmly established in reproduction, presence of *20β-HSD* transcripts in tissues such as brain, heart, muscle etc, is not very clear. Identification of receptors for 17α, 20β-DP in many tissues (Kazeto *et al*., 2005) gives clue to the wide spread distribution of CR/*20β-HSD* and it is possible that they may be involved in autocrine/paracrine/intracrinological actions. Moreover, CR activity of 20β-HSD might explain the wide range of its distribution and may be involved in the metabolism of xenobiotics and/inactivation of steroids as in mammals (Wermuth, 1981; Espey *et al*., 2000). Consistent with previous reports in tilapia and zebrafish, a low level of catfish *20β-HSD* transcript was found in liver. Since liver is the major organ involved in xenobiotic metabolism, it could be possible that *20β-HSD* may be inducible in this organ by xenobiotics. Recently, Albertsson *et al*. (2007) demonstrated the induction of trout hepatic 20β-HSD by sewage effluent.

All the earlier reports from teleosts (Guan *et al*., 1999; Senthilkumaran *et al*., 2002; Tanaka *et al*., 2002; Wang and Ge, 2002) never localized 20β-HSD in ovary or testis. Immunolocalization of 20β-HSD in regions of follicular layers of ovary in the present study validates two-cell type model proposed earlier (Nagahama, 1997). We used post-vitellogenic follicle to depict this. To confirm further during FOM, we have performed Western blotting in hCG-induced oocyte maturation, *in vitro* and *in vivo*.

Earlier study in tilapia indicated that *20β-HSD* transcript was absent in immature testis (collected from 30days old fish) and abundant in mature testis (Senthilkumaran *et al*.,

2002). Besides this study, there are no reports available on the expression pattern of *20β-HSD* both on early stages of testicular development as well as in testicular cycle. In the present study, we addressed this issue to some extent. In catfish *20β-HSD* transcript appeared at 50 days after hatching (dah) and becomes prominent by 150dah (data not shown). In testicular cycle, both *20β-HSD* transcript and protein expression gradually increased from preparatory stage to spermiating stage correlating the levels of plasma MIH that reflects the functional activity of testis with respect to spermatogenesis. However, MIH is known for final maturation in both male and female teleosts, the presence of MIH (progestin) at early stages of gonadal development was unclear. The recent discovery of the involvement of 17α, 20β-DP in meiosis of germ cells particularly in DNA replication (Miura *et al*., 2006) support our observation of expression of *20β-HSD* at early stages of testicular cycle (present study) and that of 20β-HSD activity in non-flagellated germ cells by Vizziano *et al*. (1996). Further immunoreactivity in spermatogonia and spermatocytes supports this notion to some extent. Conversely, high levels of *20β-HSD* in mature/spawning testis are required for spermiation.

In many teleosts, a massive production of MIH prior to oocyte maturation is well documented. In the present study, 17α, 20β-DP and hCG-induced GVBD response in catfish confirms that the rise in MIH is associated with FOM. However, the presence of high expression of *20β-HSD* in spawning ovary is consistence with previous finding in

trout that ovarian follicle cells acquire the 20β-HSD activity well before the onset of FOM (Kanamori *et al*., 1988; Guan *et al*., 1999). Analysis of transcript abundance of *20β-HSD* using real-time PCR approach has not been done so far. Real-time PCR and immunoblot analyses demonstrated a discrete pattern of expression of *20β-HSD*. A short-lived elevation in the *20β-HSD* transcripts seems to be sufficient enough for onset of meiotic maturation in catfish. Consistently, the expression of ovarian CR in rats distinctly increases with induction of ovulation and *20β-HSD* has been shown to increase during FOM in ayu, tilapia and murrel (Tanaka *et al*., 2002; Senthilkumaran *et al*., 2002; Chapter 1). In contrast, expression pattern of *20β-HSD* mRNA during hCG-induced oocyte maturation in zebrafish is contradictory. Kohli *et al*. (2005) reported a rise in *20β-HSD* mRNA during hCG-induced oocyte maturation while Wang and Ge (2002) demonstrated a constitutive expression pattern. This may be either due to difference in the sensitivity of techniques used or may be variation in experimental conditions. Difference in zebrafish strains might also be a reason. Therefore, the mechanism of MIH production including the activation of key enzymes involved might vary among species. In spite of the divergent actions and expression patterns of *20β-HSD*, it is considered to be important for oocyte maturation though involvement of other enzymes cannot be ruled out. Inhibition of both hCG-induced oocyte maturation and purified recombinant protein by specific chemical inhibitors in the present study further authenticate this contention. Based on our findings, we propose that ovarian

follicle acquires the 20β-HSD activity prior to induction of oocyte maturation and a marginal increase in *20β-HSD* mRNA vis-à-vis elevated enzyme production/activity might be sufficient to produce high levels of MIH that is required for FOM. On the other hand, in a natural fortnight breeder, the Nile tilapia, onset of elevation of *20β-HSD* mRNA occurs during preovulatory LH surge (Senthilkumaran *et al*., 2002). To our knowledge, perhaps different breeding pattern might be responsible for such events. This contention indeed clarifies the variable pattern of rise in *20β-HSD* transcripts in teleosts.

In summary, a full-length cDNA for *20β-HSD* was cloned from ovary and testis of catfish. Catfish *20β-HSD* cDNAs showed high homology to that of zebrafish and seems to have evolved from tilapia. Purified recombinant protein catalyzed not only the conversion of 17α-OHP but also showed carbonyl reductase activity that is inhibited by specific chemical inhibitors. Present study explicitly demonstrated the stage dependent expression of *20β-HSD* both in ovarian and testicular cycles. Several new lines of evidences including localization, responsiveness to hCG both *in vitro* and *in vivo* together with inhibition of GVBD as well as enzyme activity by carbonyl reductase specific inhibitors, demonstrate the involvement of *20β-HSD* in final meiotic maturation and also in testicular recrudescence.

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Abstract

20β-Hydroxysteroid dehydrogenase (*20β-HSD*) is the enzyme that reduces C20 carbonyl group of C21 steroids, which has broad substrate specificity and is structurally similar to mammalian carbonyl reductase1. Here we report a 2.0 kb upstream sequence of *20β-HSD* from the air-breathing catfish, *Clarias gariepinus*. Several putative transcription factor binding sites more importantly, cAMP, xenobiotic, glucocorticoid and progesterone responsive elements, AP-1, SP1, sterol regulatory element binding protein, SF-1, OCT-1, OCT-6, GATA and several other transcription factor binding sites were identified by *in silico* analysis. Luciferase reporter assays with progressive PCR based deletion mutants in Chinese hamster ovary and human embryonic kidney cell lines demonstrated that -562 region harboring CAAT box flanked by cAMP responsive element (CRE) is important for basal promoter activity. Further, increase in luciferease activity with cAMP altering drugs such as forskolin and 3-isobutyl-1-methylxanthine and specific electrophoretic mobility shift with oligonucleotides corresponding to CRE indicate the regulatory influence of cAMP on *20β-HSD* promoter activity. A cDNA encoding CRE binding protein (*CREB*) isolated from the ovarian follicles was highly homologous to vertebrate CREB1. Real-time RT-PCR analysis demonstrated synergistic expression pattern of *CREB* with that of *20β-HSD* during gonadotropin induced final oocyte maturation, *in vitro* and *in vivo*. Results from this study provide insights on the functional characteristics of *20β-HSD* promoter.

Introduction

20β-hydroxysteroid dehydrogenase (*20β-HSD*) was first reported to be present in the prokaryote *Streptomyces hydrogenans* as an enzyme reducing C20 carbonyl group of C21 steroids (Hubener and Lehman, 1958) and subsequently found in teleosts and mammals (Wermuth, 1981; Tanaka *et al*., 1991; Senthilkumaran *et al*., 2004). cDNA cloning studies have demonstrated a striking similarity in structure and function of teleost *20β-HSD* with that of mammalian carbonyl reductase1 (CBR1; Tanaka *et al*., 1991) and denoted as CBR1-like *20β-HSD* . Enzymes from both the groups of animals have a broad spectrum of substrate specificity over a wide range of xenobiotic carbonyls, endogenous steroids and prostaglandins (Wermuth, 1981; Iwata *et al*., 1990; Senthilkumaran *et al*., 2004). Fish *20β-HSD* is known to be involved in the production of 17α, 20β-dihydroxy-4-pregnen-3-one, the maturation inducing hormone (MIH; Nagahama, 1997). Up-regulation of 20β-HSD activity and transcript by gonadotropin during final oocyte maturation (FOM) has been well documented (Senthilkumaran *et al*., 2002 & 2004; Nagahama and Yamashita, 2008; Chapters 1 & 2). Besides the gonadotropin regulation, FOM is influenced by several other factors including activin (Ge, 2000), melatonin (Chattoraj *et al*., 2005), insulin like growth factor (Dilip *et al*., 2006) etc.

Although high levels of 20β-HSD was shown to be present in neonatal pig testis, its physiological significance remains unclear (Tanaka *et al*., 1991). In rodents, both inducible and non-inducible CRs have been identified (Aoki *et al*., 1997) and the

inducible form is implicated in ovulation (Espey *et al*., 2000). In humans, CR functions in phase-I xenobiotic metabolism, reduces several carbonyl compounds and CRs are involved in both physiological processes and pathological conditions (Ismail *et al*., 2000; Maser, 2006; Oppermann, 2007).

CRs are either appear to be house keeping genes or regulated via endogenous hormones and growth factors (Oppermann and Maser, 2000). Rodent CRs and teleost *20β-HSD*s are inducible by gonadotropins (Inazu *et al*., 1992; Senthilkumaran *et al*., 2002; Chapters 1 & 2) while human gene responds to classical xenobiotic inducers e.g. napthoflavone, phenobarbital etc. and functional xenobiotic response elements were identified in human CBR1 gene promoter (Lakhman *et al*., 2007). However, factors that regulate rodent CRs and other vertebrate *20β-HSD* expression apart from gonadotropin are yet to be identified. Hence, functional characterization of *20β-HSD* promoter may shed light on our understanding of regulation of CR/*20β-HSD* expression in view of their roles in both reproductive and non-reproductive processes.

In the present study, we isolated upstream sequence of the air-breathing catfish, *Clarias gariepinus 20β-HSD*. The putative cis-acting elements were identified by *in silico* analysis and functional analysis of the promoter was carried out by PCR based progressive deletion mutants. In addition, we present the evidences for the regulation of catfish *20β-HSD* gene expression by cAMP.

Materials and Methods

Cell culture and reagents

HEK-293 (human embryonic kidney) and CHO (Chinese hamster ovary) cell lines were obtained from the National Centre for Cell Science (Pune, India). Minimum essential medium (α–MEM), fetal bovine serum and other cell culture reagents were purchased from Gibco-BRL (Invitrogen, Carlsbad, CA, USA). Cells were routinely cultured in 75 cm^2 vented flasks using α-MEM supplemented with 10% fetal bovine serum. Cultures were grown in an incubator at 37°C, 5% CO_2 and 95% relative humidity. Cultures were maintained at low passage numbers (n<10). Forskolin and 3-isobutyl-1-methylxanthine (IBMX) were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Construction of Genome walking library and isolation of upstream elements

Genomic DNA was isolated from the ovarian follicles of catfish using genomic DNA isolation kit (Bangalore Genei, Bangalore, India) following manufacturer's protocol. Genome walking library was constructed using universal genome walker kit (Clontech, Mountainview, CA, USA) following manufacturer's instructions. Twenty five μg of genomic DNA was digested overnight each with *Eco*RV, *Pvu*II, *Dra*I and *Stu*I. Digested DNA was purified using phenol chloroform extraction method. Genomic DNA digested with each restriction enzyme was ligated to adaptors separately. *20β-HSD* upstream region was isolated using gene specific primers (Table 1) designed in the first exon of the *20β-HSD* and the adapter primer-1. Touchdown PCR was used for the

amplification. Cycling conditions were 94°C 30sec, 72°C 3 min, 5 cycles, 94°C 30sec, 68°C 30sec, 72°C 3 min for 30 cycles. A nested PCR was carried out using gene specific primers (Table 1) and adaptor primer-2 (both the adapter primers were given in the genome walker kit), all the amplicons were cloned into pGEM-T Easy vector (Promega, Madison, WI, USA) and sequence was determined bi-directionally.

Primer	Sequence 5' – 3'	Purpose
GSP-R1	CGTCGCGCGCCATTAGGTACACGTC	Genome walking, PCR
GSP-R2	CGTGACCTGCGAGCACAAGACACGC	Genome walking, PCR
F1	GGTACCATGCACTCCTAACACTTTAGCG	-1971/+42 (deletion mutant)
F2	GGTACCACTTCACTTCACCCCTTCACTG	-1474/+42 (deletion mutant)
F3	GGTACCACTTCAATAACTTCACATTCTG	-803/+42 (deletion mutant)
F4	GGTACCTCCGAGTAGGTACTCCTCACAG	-562/+42 (deletion mutant)
F5	GGTACCACTCCGGTTTCCTCCCACAG	-384/+42 (deletion mutant)
F6	GGTACCCTCCCACCTACGGATGGCTG	-210/+42 (deletion mutant)
R	AAGCTTGACCTGCGAGCACAAGACACGC	Common reverse primer (deletion mutants)

Table 1. List of primers used for catfish *20β-HSD* upstream sequence cloning and promoter analysis.

In silico analysis

Core promoter prediction was carried out using the software neural network promoter prediction program. Putative transcription factor binding sites were predicted using the programs such as MatInspector and TRANSFAC.

Cloning of *20β-HSD* promoter deletion constructs

Progressive 5' deletion constructs were made by PCR using the primers listed in table 1. The resulted deletion mutants were cloned in *Kpn*I and *Hind*III sites of pGL2 basic firefly luciferase vector (Promega). The identity of each construct was verified by double digestion and the absence of cloning artifacts was determined by nucleotide sequencing.

Transient transfections and luciferase reporter activity assays

Cells were plated 24-48 hours before transfections in 24 well plates. Reporter gene constructs (firefly luciferase), and the *Renilla* luciferase pRL-TK plasmid were co-transfected into 60 - 70% confluent cell cultures by using Tfx20 (Promega). Forty-eight hours after co-transfections, cultures were washed once with phosphate buffered saline solution and the cells were lysed with 100 µl per well passive lysis buffer (Promega). Cell lysates were incubated at room temperature (15 min), mixed with a vortex blender (10 sec), and centrifuged at 4°C (1500 rpm, 30 sec). Luciferase reporter activities were determined with the dual-luciferase reporter assay system (Promega) according to the

manufacturer's instructions. Light intensity was measured using Turner Design 20/20 luminometer. Luciferase reporter activities were expressed as relative with respect to the values obtained for *Renilla* luciferase.

Molecular cloning of *CREB* from catfish ovary

A set of degenerate primers designed (Table 2) by aligning other vertebrate *CREB* cDNA sequences were used to amplify a partial cDNA fragment by RT-PCR. Gene specific primers (Table 2) for 5' and 3' rapid amplification of cDNA ends (RACE) were designed from partial cDNA sequence and RACE was performed to isolate 5' and 3' ends of catfish *CREB*. Methodologies used for obtaining partial cDNA and RACE were described in chapter 2.

Northern blotting

Twenty five micrograms of total RNA prepared from different stages of ovarian follicles was analyzed by Northern blotting. Catfish *CREB* open reading frame (ORF) cDNA was used to probe the membranes and method followed was described in detail in chapter 2.

Real-time RT-PCR

Transcript abundance of *CREB* during human chorionic gonadotropin (hCG)-induced oocyte maturation, *in vitro* and *in vivo* as well as ovary at different stages of gonadal

cycle was done by real-time RT-PCR using gene specific primers (Table 2) following the methodology as described in chapter 2.

Primer	Sequence 5' – 3'	Purpose
DF1	CATMTATCAGACYAGCASSGGSCA	Degenerate RT-PCR
DR1	CYTTCTTCTTCCTGCGACACTC	Degenerate RT-PCR
GSP-R1	AGTTTGCAGCCCTTGCACGCCGTC	5' RACE
GSP-R2	CTGGATGGCTCCACCCTGTGTGAT	5' RACE
GSP-F1	CGCCTCATGAAGAACAGGGAAGC	3' RACE
GSP-F2	AGGGAAGCGGCCCGAGAGTGTCGC	3' RACE
ORF-F	GCTAGCATGACCATGGAGGCGGGAGCGGAG	ORF cloning
ORF-R	CTCGAGTTACTCGGATTTATGGCAGTACAG	ORF cloning
qRT-F	CGTCCTTCTTACAGGAAGATCC	Real-time RT-PCR
qRT-R	TCTCTGAGCTGTATTTGGCACG	Real-time RT-PCR

Table 2. Primers used for *CREB* cloning and expression.

Electrophoretic mobility shift assay (EMSA)

Gel shit assay was performed as per the method described previously (Yoshiura *et al*., 2003). Oligomeric sequence of catfish *20β-HSD* region harboring a cAMP responsive element (CRE) and a mutated CRE were synthesized and labeled by [^{32}P]dATP using polynucleotide kinase. The labeled oligonucleotide probe was added to 20 µl binding

reaction with 5 µg protein of catfish ovarian follicular nuclear extract. Specificity of binding was assessed using un-labeled double stranded DNA oligos as competitors. Binding reactions were incubated on ice for 45 min and DNA-protein complexes were separated on 8% non denaturing polyacrylamide gel at 10 mA for an hour. Gels were dried and visualized using phosphorimager (Typhoon, GE Healthcare).

oligo	Sequence 5' – 3'
CRE	CTGTCGTTTTGGCTGACGTCACTGTCCCCCAGAGGACGTC
CRE	GACGTCCTCTGGGGGACAGTGACGTCAGCCAAAACGACAG
CRE-mutant	CTGTCGTTTTGGCTAAAGTCACTGTCCCCCAGAGGACGTC
CRE-mutant	GACGTCCTCTGGGGGACAGTGACTTTAGCCAAAAGACAG

Table 3. Native and mutant CRE oligonucleotide seuences used in EMSA. CRE region is underlined.

Data analysis

All the real-time RT-PCR and luciferase reporter activity assays were done three times independently in triplicates and data was expressed as ±SEM. Statistical analysis was done using Sigmastat 3.1 software. Significance between different groups was tested by ANOVA followed by Student's-Neuman-Keul's test. Difference between groups were considered at $P<0.05$.

Results

Cloning and *in silico* analysis of *20β-HSD* promoter

A 2.0 kb upstream sequence of catfish *20β-HSD* was obtained by genome walking approach (Fig. 1). Transcriptional start point (TSP) was determined by 5' RACE (chapter 2) and found to be at 59 nucleotides upstream of ATG start codon. Analysis of TSP by neural network promoter prediction program also identified TSP at the same location. Analysis of core promoter motifs identified a non-canonical TATA box (TAATAAA). CpG islands in the proximal promoter region were also predicted with an observed/expected ratio greater than 0.6 but %C+G was marginal (Fig. 2)

Computer assisted searches using the programs MatInspector and TRANSFAC for additional cis-acting elements indicated putative consensus motifs for several transcription factors. Those with maximum core and matrix similarity include activator protein1 (AP-1), specificity protein1 (SP1), steroidogenic factor1 (SF-1), octamer binding proteins 1 & 6, (OCT-1 & OCT-6), GATA binding factor (GATA), CAAT binding factor (CBF), TATA box binding protein (TBP), hepatic nuclear factor (HNF) glucocorticoid, progesterone responsive elements (GRE & PRE). More importantly, cAMP and xenobiotic responsive elements (CRE & XRE) were observed in both proximal and distal ends of the promoter (Fig. 2).

Functional analysis of *20β-HSD* promoter constructs

We generated a series of progressive PCR based deletion mutants (Figs. 3 & 4) and performed gene reporter assays to identify the regions important for regulation of *20β-HSD* expression. Results from the reporter assays indicated the presence of negative regulatory elements in the region of -1474 to -803 as there was a significant decrease in reporter activity from -1971/+42 construct. A considerably high reporter activity was found with -562/+42 construct that has a CAAT box flanked by CRE. Deletion of CRE decreased promoter activity significantly. Luciferase reporter activities were more or less similar in both *HEK293* and *CHO* cell lines (Fig. 5).

Effect of forskolin and IBMX on *20β-HSD* promoter activity

To implicate the functionality CRE, we used different doses of forskolin, drug that increase cellular cAMP with a fixed concentration of IBMX (phosphodiesterase inhibitor) in gene reporter assays. Forskolin at 0.5 μM concentration strongly induced the promoter activity with full-upstream region as well as with -562/+42 promoter construct while the deletion of CRE did not show significant difference in promoter activity (Fig. 6).

Identification of CREB binding site

Gel shift assay using ovarian follicular nuclear extracts demonstrated a complex formation with ^{32}P labeled oligomeric sequence containing CRE motif while no

complex was observed with mutated oligo. Inability of nuclear extracts in the presence of cold competitor oligo indicates the specificity of binding (Fig. 7).

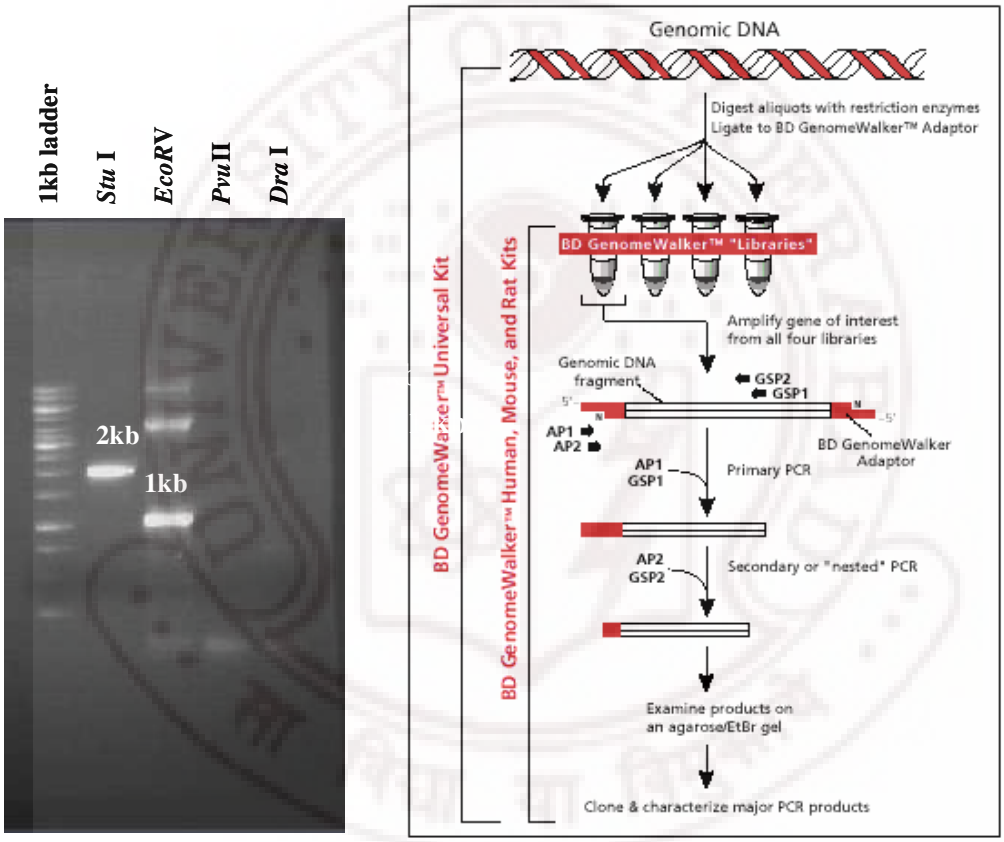


Fig. 1. 1% Agarose gel showing the nested PCR amplifications using *20β-HSD* gene specific primers in catfish genome walking library and right side panel is schematic representation of genome-walking strategy.

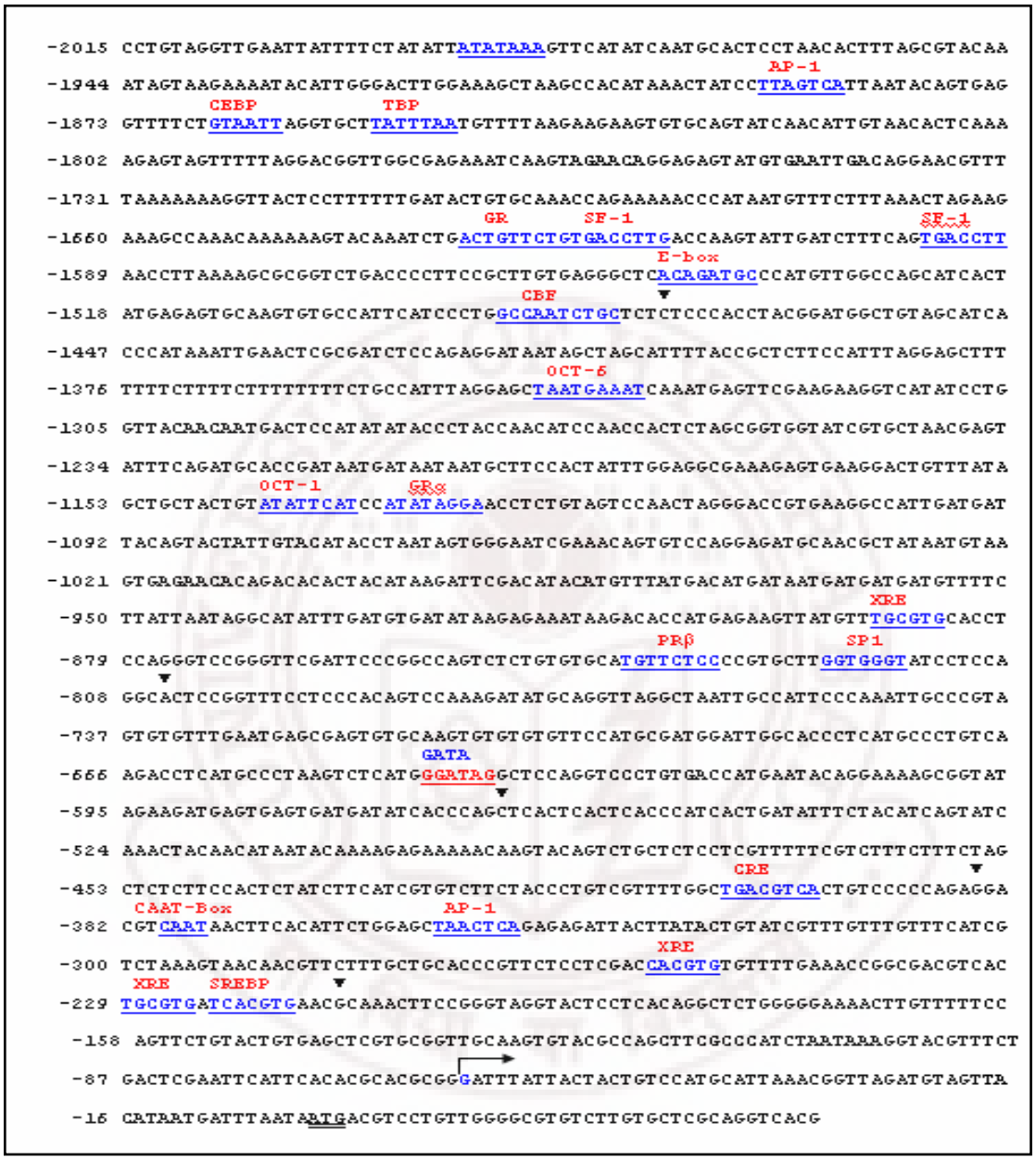


Fig. 2. Nucleotide sequence from the 5' flanking region of catfish *20β-HSD*. The translational start site is double under lined and the transcriptional start site is indicated with an arrow and bold face letter. The different fragments corresponding to the series of progressive deletion promoter constructs are indicated with downward arrow. The putative transcription factor binding sites are underlined and shown in different colors.

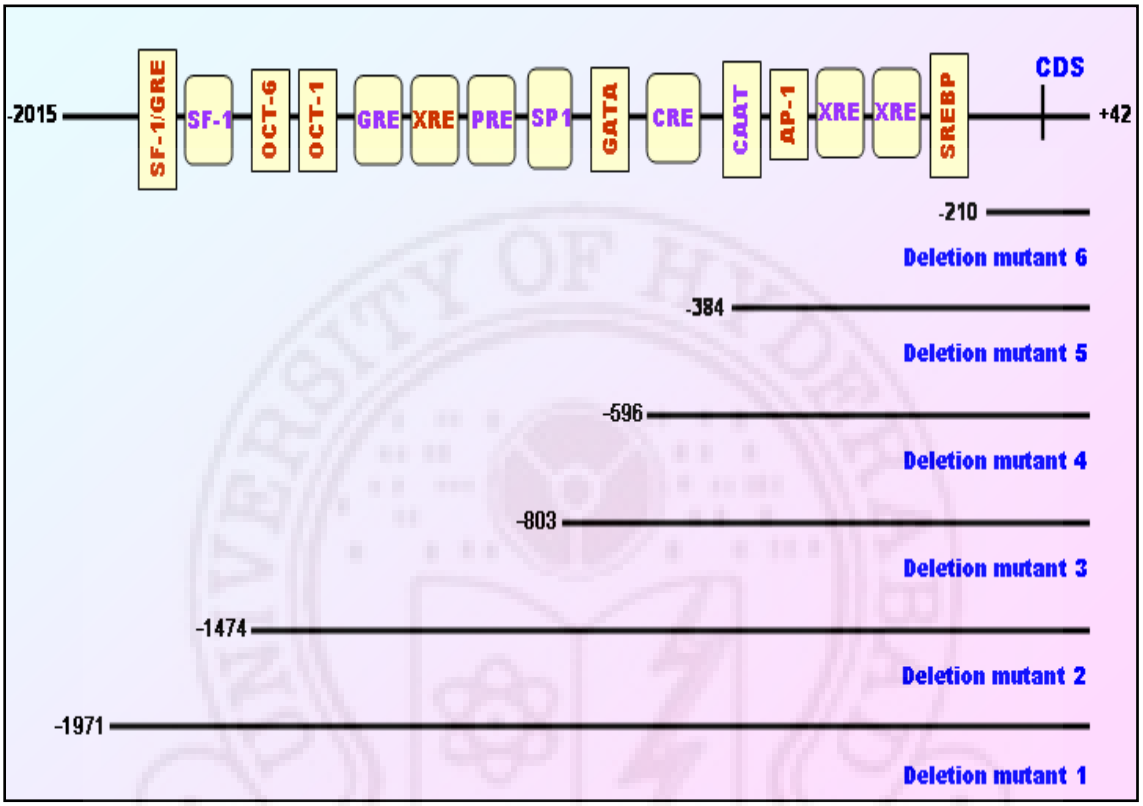


Fig. 3. Schematic representation of catfish *20β-HSD* promoter and PCR based deletion mutants. CDS stands for coding sequence.

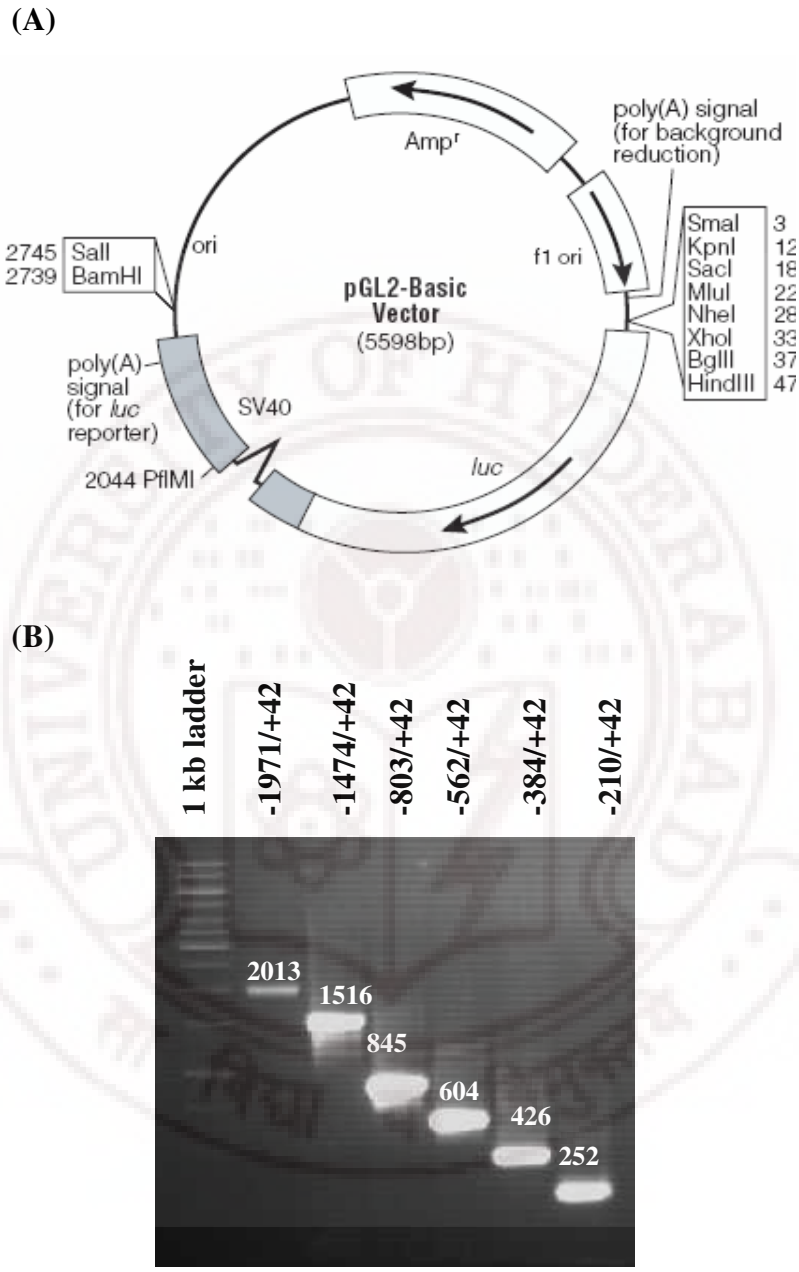


Fig. 4. Map of luciferase reporter vector pGL2 (A). A 1% agarose gel showing the PCR amplicons of deletion mutants (B). Numbers over each band represents the size of band in base pairs.

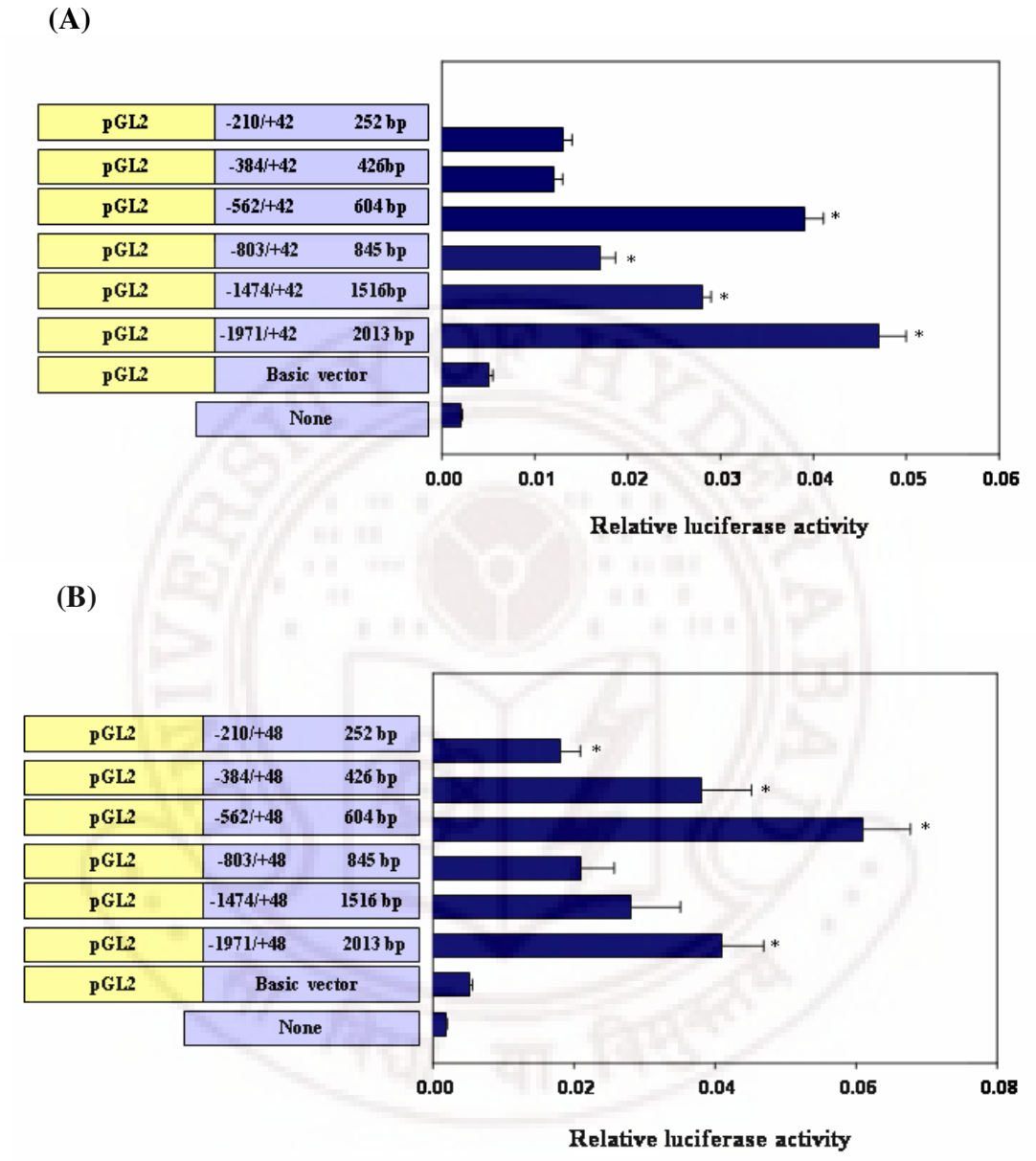
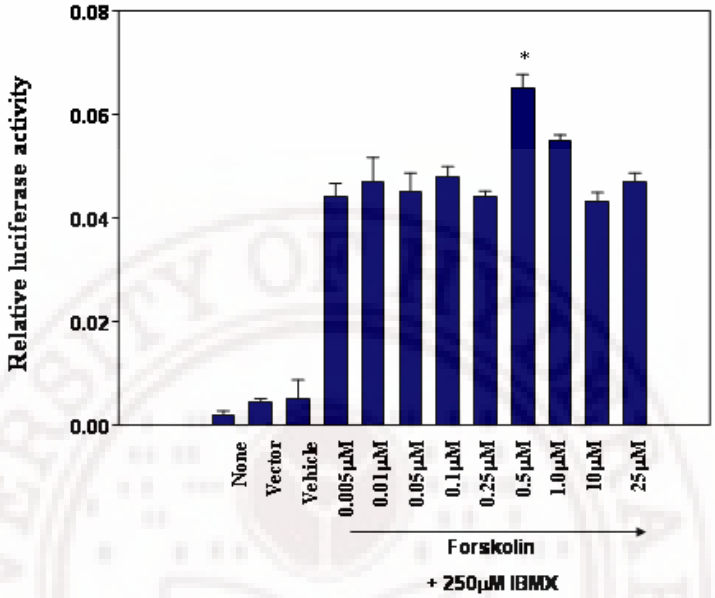


Fig. 5. Functional analysis of different *20β-HSD* promoter constructs (indicated on the left side) co-transfected with pRL-TK into HEK293 (A) and CHO (B) cells. Luciferase activity is presented as relative to the activities measured for *Renilla* luciferase (∗ indicates significance ANOVA, $P<0.05$).

(A)



(B)

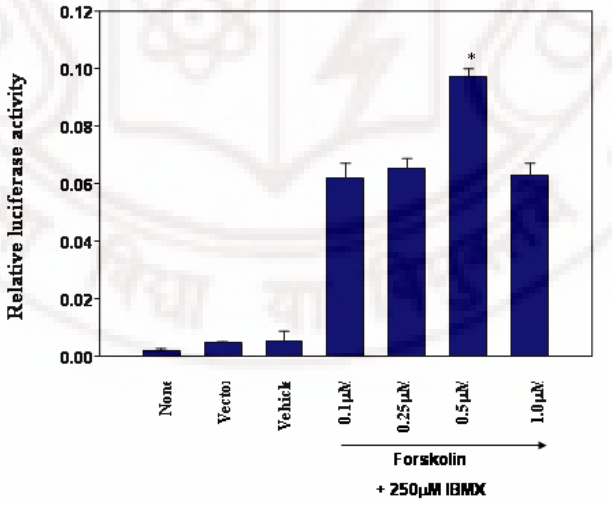


Fig. 6. Effect of cAMP altering drugs on the luciferase reporter activity of catfish *20β-HSD* promoter in CHO cells with -1971/+42 (A) and -562/+42 (B) promoter constructs (∗ indicates significance, ANOVA, *P*<0.05).

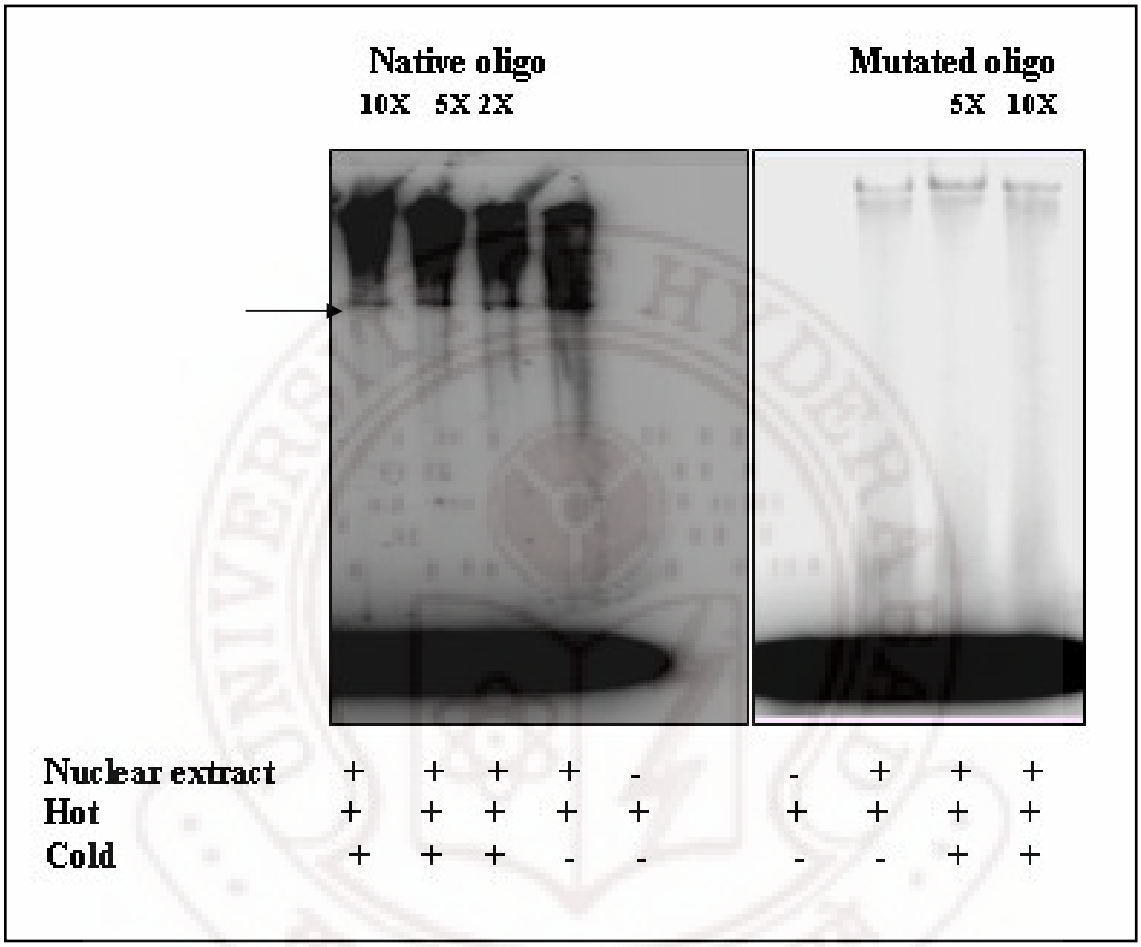


Fig. 7. Autoradiogram showing the electrophoretic mobility shift of follicular nuclear extracts incubated with oliconucleotide sequence corresponding to predicted CRE. Same oligonucleotide with mutated sequence was used as negative control. Un-labeled oligonucleotides were used in indicated fold excess for competition.

Molecular cloning of CREB from catfish ovary

A partial cDNA of 405 bp was obtained from the ovarian follicles using a set of degenerate primers by RT-PCR. The identity of cloned cDNA was confirmed by BLAST search. Full-length cDNA of *CREB* was isolated from the catfish ovarian follicles by 5' and 3' RACE strategies (Fig. 8) using gene specific primers designed from partial cDNA clone (Table 3). Full-length catfish *CREB* cDNA was 1.398 kb with an ORF of 975 bp encoding a protein of 375 amino acids. The 5' UTR was 91 bp while 3' UTR was 332 bp (Fig. 9). The putative protein is highly conserved encompassing signature domains, kinase inducible and DNA binding domain (bZIP). Catfish ovarian *CREB* shares highest homology with that of zebrafish (79%). Phylogenetic analysis by ClustalW method demonstrated that catfish *CREB* segregated with zebrafish into a separate clade, where as tilapia *CREB*s formed into distinct clade (Fig. 10).

Expression of *CREB* in ovary

Real-time RT-PCR analysis demonstrated that transcript abundance of *CREB* was high in the preparatory and spawning phases while it was low in pre-spawning and regressed phases of ovarian cycle (Fig. 11). Northern blot analysis of *CREB* identified a single transcript of about 1.4 kb and the expression in different stages of ovary by Northern blot analysis was in accordance with real-time RT-PCR results (Fig.11). In hCG-induced oocyte maturation, *in vitro*, *CREB* mRNA levels increased by four hours after treatment with hCG, reached a peak level by 6 hours and there by decreased (Fig. 12).

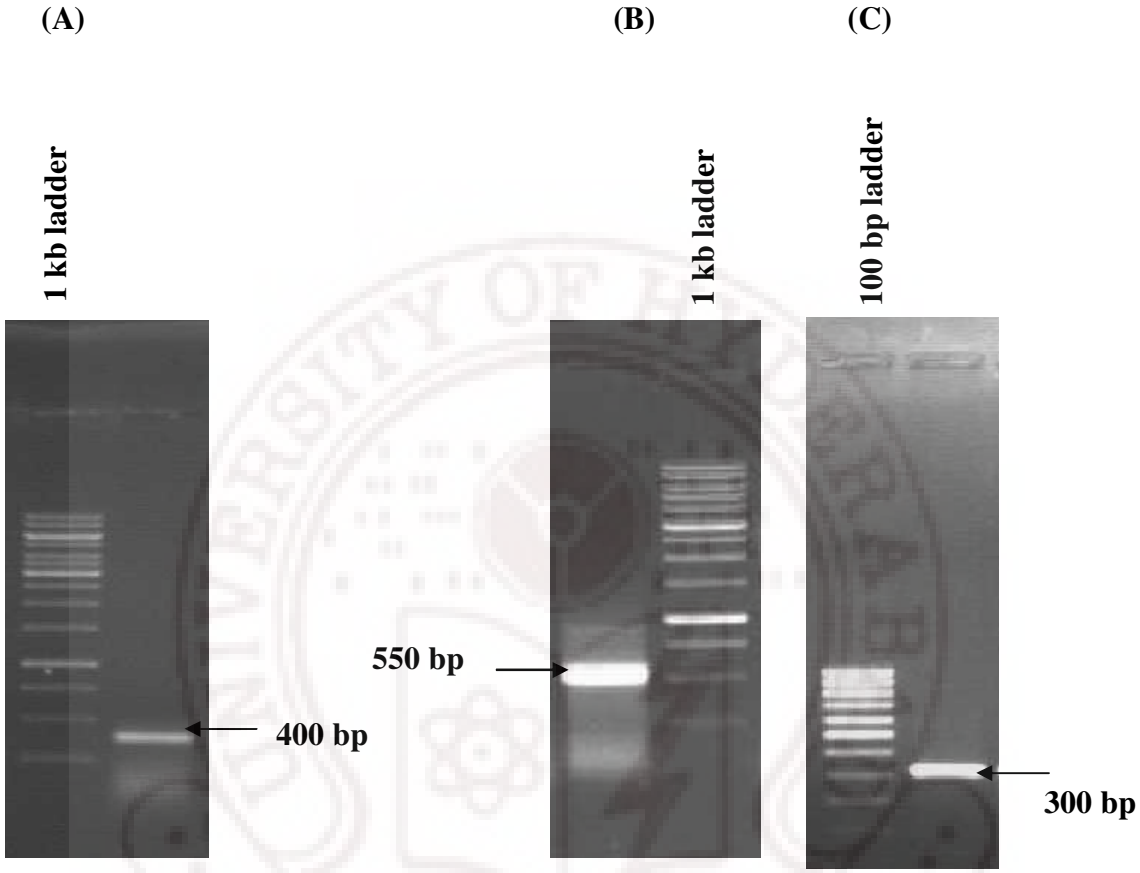


Fig. 8. 1% Agarose gels showing RT-PCR products using degenerate primers for amplification of partial *CREB* cDNA fragment (A), 5' and 3' RACE (B & C) products using gene specific primers to obtain *CREB* full-length cDNA.

```
ggacactgacatggactgaaggagtagaaag                                        31
tataatttcgtacaccctcgagctcacttcggccaccttcgtgtgctgttcgataactca 91
atgaccatggaggcgggagcggaggcccagcagggtgcagacacggctgtggctgagact 151
 M  T  M  E  A  G  A  E  A  Q  Q  G  A  D  T  A  V  A  E  T
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 V  Q  V  H  G  V  I  Q  A  A  Q  P  S  V  I  Q  S  P  Q  V
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 V  T  D  S  Q  K  R  R  E  I  L  S  R  R  P  S  Y  R  K  I
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 H  K  S  E  *
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ggaaaacacatattcatgtgttgctttagaaaaaaaaaaaaaaaaa                 1398
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Fig. 9. Nucleotide (blue) and deduced amino acid (red) sequence of catfish ovarian *CREB*. UTRs are shown in black letters and polyadenylation signal is shown in boldface letters with underline.

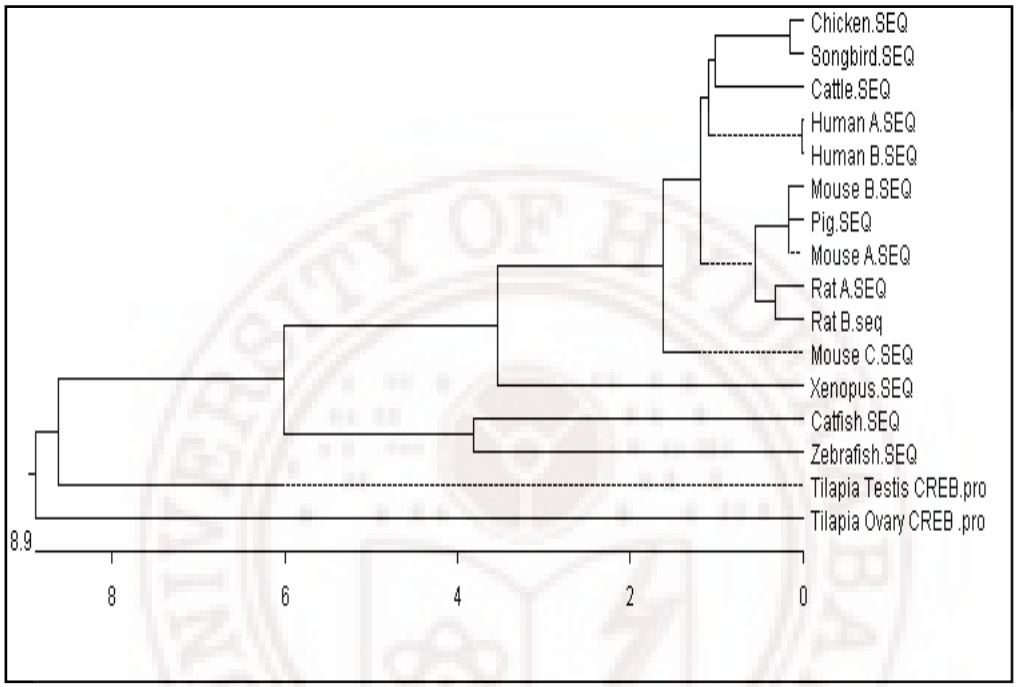
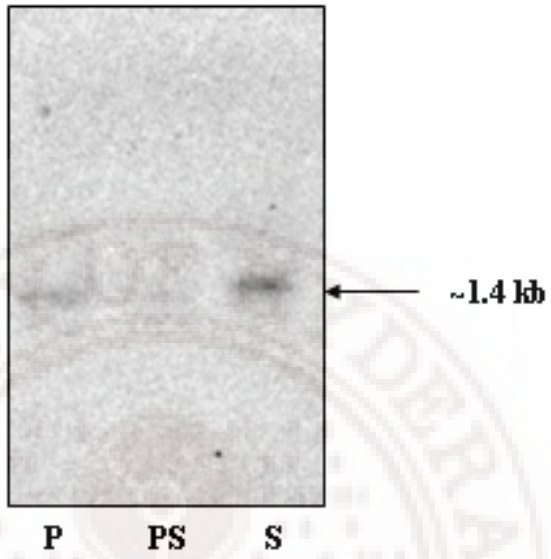


Fig. 10. Phylogenetic tree showing the evolutionary status of catfish *CREB*. (Accession no. : Human A **NM_004379**; Human B **NM_134442**; Mouse C **NM_001037726**; *Xenopus* **NM_001086603**; Mouse A **NM_009952**; Mouse B **NM_133828**; **Rat A NM_ 134443**; Rat B **NM_031017**; Chicken **NM_ 204450**; Zebrafish **NM_ 200909**; Pig **NM_001099929**; Cattle **NM_174285**; Songbird **NM_001048256**).

(A)



(B)

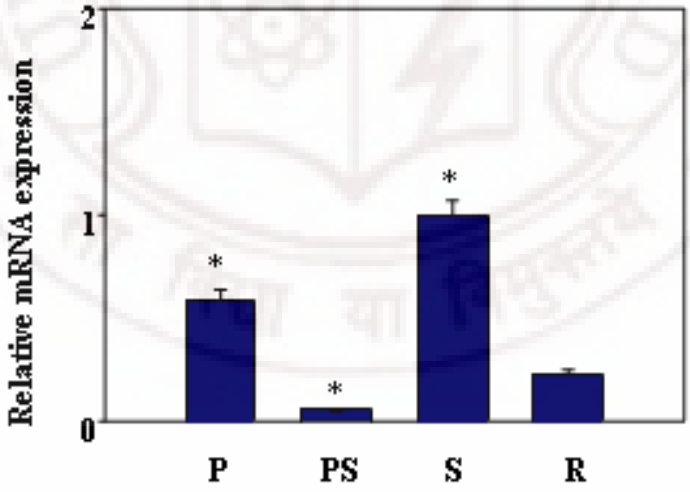
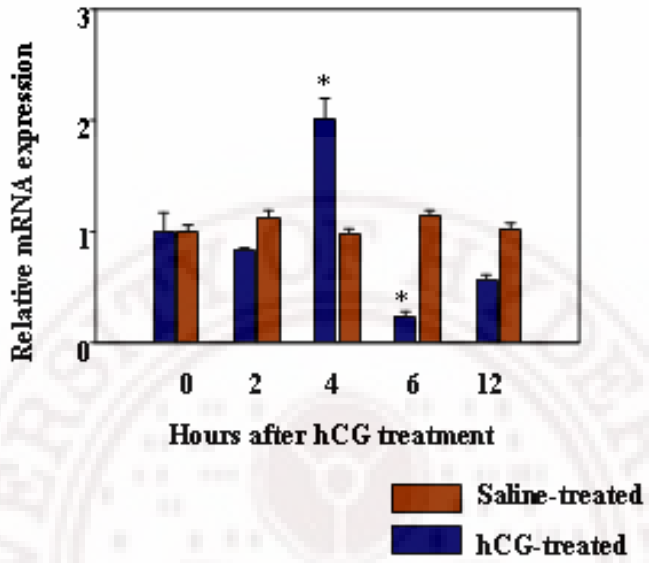


Fig. 11. Expression of *CREB* in different stages of catfish ovary by Northern blot (A; representative, n=3) and real-time RT-PCR analysis (B). P, preparatory; PS, pre-spawning; R, regressed (∗ indicates significance, n=3, ANOVA, $P<0.05$).

(A)



(B)

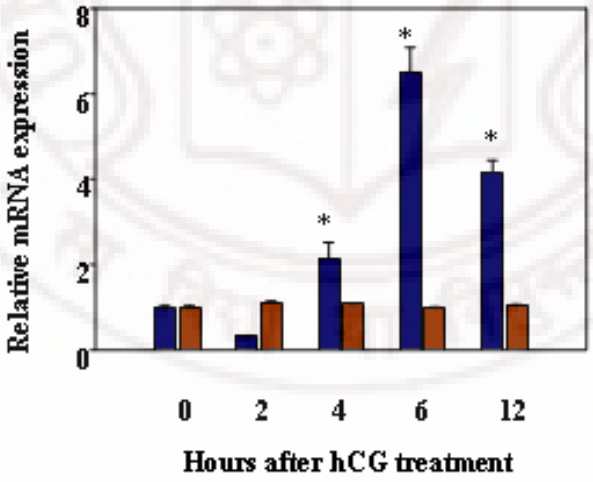


Fig. 12. Real-time RT-PCR analysis of *CREB* expression during hCG-induced oocyte maturation, *in vitro* (A) and *in vivo* (B). ∗ Indicates significance (n=3, ANOVA, *P*<0.05).

Discussion

In the present study, we report a 2.0 kb up stream region of *20β-HSD* from catfish. *20β-HSD* has been identified both in mammals and teleosts, but reports on transcriptional regulation of *20β-HSD* are very much limited (Senthilkumaran *et al*., 2001). However, promoter of CBR1 from rat and humans has been isolated (Aoki *et al*., 1997; Lakhman *et al*., 2007). Though these enzymes shares very high similarity in the structure and enzymatic activity, but they involve in different physiological processes and consistently their promoter organizations are also different. Human and mouse carbonyl reductase promoters have features of a prototypical CpG promoter with GC boxes and either TATA box or down stream promoter elements are absent (Aoki *et al*., 1997; Lakhman *et al*., 2007). In contrast, *20β-HSD* promoter has a TATA box while CpG islands were predicted in the proximal region marginally. Our results with gene reporter experiments in HEK293 and CHO cells demonstrated the presence of regulatory elements in -562 region that appear to relevant to promote transcription at basal condition.

The orphan nuclear receptor Ad4BP/SF-1 is important for development and function of steroidogenic organs. Many studies have highlighted important roles for Ad4BP/SF-1 in transcriptional regulation of several steroidogenic enzymes both in teleosts and higher vertebrates (Ikeda *et al*., 1994; Yoshiura *et al*., 2003; Achermann, 2005). Presence of Ad4BP/SF-1 sites at -1519/-1525 region in catfish *20β-HSD* promoter indicates possibility for the regulation of this enzyme by Ad4BP/SF-1. However, studies from

tilapia *20β-HSD* promoter activity indicated no regulatory role for Ad4BP/SF-1 (Senthilkumaran *et al*., 2001 & 2004).

GATA are members of zinc finger transcription factor family that regulate target gene transcription through a common DNA binding motif (A/T)GATA(A/G). GATA-4 and 6 are known to be important for development as supported by the deficient mice die during early development. An important role for GATA-4 in gonadal expression of steroidogenic enzyme genes has been suggested by several studies and GATA-4 is shown to work synergistically with Ad4BP/SF-1 (LaVoie, 2003). Since *20β-HSD* expression is known to be associated with differentiation of spermatogonia (Miura *et al*., 2006), presence of GATA binding site on *20β-HSD* promoter indicates that this also may be one of the pathways in gonadal development in fish.

Expression of *20β-HSD* is ubiquitous in teleosts, though their role is not well defined in tissues except for gonads. Observation of low expression of *20β-HSD* in liver (Senthilkumaran *et al*., 2002; Chapter 2) and its induction by sewage effluents in liver of trout (Albertson *et al*., 2007) together with high specific activity of *E. coli* expressed recombinant 20β-HSD proteins on xenobiotics (Senthilkumaran *et al*., 2002; Chapter 2) provides impetus to the hypothesis that *20β-HSD* may also be involved in xenobiotic metabolism like that of mammalian CBR1s. Further identification of XRE both in catfish *20β-HSD* promoter as well as human CR gene potentiates the role of these enzymes in xenobiotic metabolism.

We observed GRE and PRE in the promoter of *20β-HSD*. Though we did not perform assays relating to the modulation of *20β-HSD* expression by steroids, few studies are available on this line. Similar to the one that occurs in teleost ovarian follicles, a shift in steroidogenesis from androgens to progestins was also demonstrated in spermiating male fishes (Barry *et al*., 1990) and it is believed that estradiol has a role to play in increasing the levels of MIH with the onset of spermiation (Vizziano *et al*., 1996), probably through feedback regulation. Imamura *et al*. (2001) demonstrated regulation of *20β-HSD* activity by testosterone in rat and observation of sex steroid hormone responsive elements on *20β-HSD* promoter provides the direct evidence for hormonal regulation of *20β-HSD*.

cAMP responsive genes are known to be regulated by cues such as extracellular stresses or hormonal signals (Sands and Palmer, 2008). We identified a CRE element in the -328 region of *20β-HSD* promoter. Luciferase reporter assays have demonstrated that promoter construct of this region is important for *20β-HSD* promoter activity. Inducibility of the promoter constructs with cAMP enhancing drugs support the notion of *20β-HSD* expression during oocyte maturation in teleost species (Senthilkumaran *et al*., 2004; Chapters 1 & 2).

CREB members represent a large family of bZIP transcription factors rather with diverse physiological functions. CREB members are both ubiquitously expressed and/or tissue specifically expressed, with latter controlling cell specific pattern of gene transcription (Sands and Palmer, 2008). Though the presence of multiple forms of

CREBs within a particular cell type is very common (Senthilkumaran *et al*., 2004) owing to its diverse array of functions, we could able to isolate a single type of *CREB* from the ovarian follicles of catfish. Synergistic expression pattern of *CREB* with that of *20β-HSD* in catfish might support the observation of *20β-HSD* mRNA surge upon stimulating with hCG. Although our finding that CRE regulates *20β-HSD* promoter is not surprising, given its extensive role as transcriptional regulator, our results define that *20β-HSD* gene is responsive to cAMP.

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Abstract

Role of cytochrome P450 17α-hydroxylase/c17-20 lyase (*P450c17*) in the shift in steroidogenesis during oocyte maturation in teleosts is a contentious issue even after identification of a novel type of *P450c17* that lacks lyase activity. To understand the role of *P450c17* in steroidogenic shift explicitly, a full length cDNA encoding *P450c17* from ovary of air-breathing catfish, *Clarias gariepinus* was cloned. Transient transrection of *P450c17* in COS-7 cells converted progesterone to androstenedione through 17α-hydroxyprogesterone and catfish *P450c17* was found to express ubiquitously with relatively higher levels in gonads, brain, kidney and gills. *P450c17* expression and ratio of lyase to hydroxylase was high in the preparatory and pre-spawning phases of ovary and low in spawning phase. Expression of *P450c17* correlated well with testicular recrudescence with a maximum expression in the preparatory and spawning phases. Neither protein expression nor lyase/hydroxylase activity changed significantly during human chorionic gonadotropin-induced oocyte maturation, *in vitro* and *in vivo* though mRNA levels increased. These results tend to suggest that the ovarian follicles attains capacity to produce maximum precursor steroid levels before spawning that might contribute to the shift in steroidogenesis.

Introduction

Cytochrome P45017α-hydroxylase/c17-20 lyase (P450c17) is a microsomal enzyme that catalyzes two distinct activities; the 17α-hydroxylase activity converts pregnenolone or progesterone to 17α-hydroxypregnenolone or 17α-hydroxyprogesterone (17α-OHP) and c17-20 lyase activity breaks the C17-20 bond of C21 steroids 17α-hydroxypregnenolone or 17α-OHP to produce dehydroepiandrosterone (DHEA) or androstenedione (AD), respectively (Nakajin *et al*., 1981). Thus, P450c17 controls an important branch point in steroid hormone biosynthesis leading to the production of three classes of main steroid hormones namely glucocorticoids, mineralocorticoids and precursors of sex steroids (Payne and Hales, 2004).

P450c17 catalyzes two mixed function oxidase reactions utilizing cytochrome P450 oxidoreductase and microsomal electron transfer system. These reactions require NADPH and molecular oxygen (Payne and Hales, 2004). Lyase activity is regulated by a set of modulators in tissue dependent manner. In mammals, modulators of lyase activity include electron-donating redox partners such as P450 reductase (Auchus and Miller, 1999). Cytochrome b5 has been shown to stimulate lyase activity by acting as an allosteric facilitator (Soucy *et al*., 2003). Moreover, serine/threonine phosphorylation of P450c17 increases the affinity of enzyme for redox partners (Jhang *et al*., 1995; Miller *et al*., 1997) and is implicated in adrenarche, polycystic ovarian syndrome and associated insulin resistance in humans (Jhang *et al*., 1995).

cDNA encoding *P450c17* has been cloned from several higher vertebrates including human (Chung *et al*, 1987), bovine (Zuber *et al*., 1986), pig (Zhang *et al*., 1992), chicken (Ono *et al*., 1988), rat (Fevold *et al*., 1989) and mouse (Youngblood *et al*., 1991). *P450c17* expression is regulated by gonadotropins (Voutilainen *et al*., 1986; Voss and Fortune, 1993), adrenocoriticotropin (Zuber *et al*., 1986), growth factor such as transforming growth factor and insulin like growth factor-I (Magoffin and Weithman, 1993; Carr *et al*., 1996), activin, inhibin (Sawetawn *et al*., 1996) and anti-mullerian hormone (Matt Laurich *et al*., 2002). On the other hand, hormonal regulation of *P450c17* in lower vertebrates including teleosts remains to be clarified.

During final oocyte maturation (FOM), a shift in steroidogenesis from estradiol-17β to 17α, 20β-dihydroxy-4-pregnen-3-one (17α, 20β-DP) is demonstrated for several teleost species (Nagahama, 1997; Joy *et al*., 1998) and reports do exist on high expression of 20β-hydrxoysteroid dehydrogenase (Senthilkumaran *et al*., 2004; Chapter 1 & 2), enzyme that produces 17α, 20β-DP. However, it is possible that *P450c17* might play a crucial role in shift in steroidogenesis by controlling the availability of precursor steroids. Though there are reports available on the cloning and expression of *P450c17* from different fish species (Sakai *et al*., 1992; Trant, 1995; Kazeto *et al*., 2000; Halm *et al*., 2003; Wang and Ge, 2004), none of these reports neither recorded changes in expression pattern and protein level/activity during steroidogenic shift nor provided convincing claim of regulation of lyase activity. But very recently, a novel form of *P450c17* that lacks lyase activity has been identified in tilapia (a fortnight breeder) and

medaka (a daily breeder) whose differential expression pattern was proposed to be important during shift in steroidogenesis (Zhou *et al*., 2007a & b). However, many teleost species that reproduce annually (more importantly fresh water inhabitants) possess only single form of *P450c17* (*P450c17-I*) and its role during meiotic maturation is a contentious issue. Against this backdrop, present study is designed to delineate contribution of *P450c17* expression and activity in steroidogenic shift. *P450c17* was cloned and characterized from the ovarian follicles of air-breathing catfish, *Clarias gariepinus*. Expression of *P450c17* during human chorionic gonadotropin (hCG)-induced oocyte maturation *in vitro* and *in vivo* was analyzed by quantitative real-time RT-PCR and Western blot methods. In addition, expression in testicular recrudescence and tissue distribution pattern was analyzed. Results from this study provide interesting information about the role of *P450c17* in steroidogenic shift during FOM.

Materials and methods

Animals and treatments

Adult catfish weighing about 400-500 g were purchased live from local fish markets (Hyderabad, India). Maintenance of animals and hCG-induction was already described in chapter 2.

Molecular cloning of catfish *P450c17*

A set of degenerate primers were designed by aligning the existing sequences of teleost *P450c17* to clone a cDNA fragment from the ovarian follicles of catfish. After obtaining a partial cDNA of *P450c17*, 5' and 3' sequences were cloned following rapid amplification of cDNA ends (RACE) approach using gene specific primers designed from partial cDNA (Table 1). Details of methodology followed for cloning was already described in chapter 2.

Genomic Southern and Northern blot analysis

Southern analysis of genomic DNA prepared from ovarian follicles and Northern blot analysis of total RNA prepared from spawning ovary and tesis was carried out by following methodology described in chapter 2 using a ^{32}P- labelled 450 bp partial cDNA of *P450c17*.

Production of rabbit anti-catfish P450c17 antiserum

To produce catfish P450c17 antigen, a 1.365 kb *Nde*I-*Xho*I fragment of catfish ovarian *P450c17* cDNA lacking the region encoding the N-terminal 56 amino acids was inserted into the *Nde*I and *Xho*I sites of vector pET28a (Novagen, La Jolla, CA, USA). The expression constructs were verified by restriction analysis and checked for cloning artifacts, if any by nucleotide sequencing. Expression, purification of recombinant P450c17 and production of polyclonal antiserum was carried out following methods described in chapter 2.

Primer	Sequence 5' – 3'	Purpose
DF1	ASCTGCARMAGAARTAYGG	Degenerate RT-PCR
DR1	CACYTCYCTGATRGTGGCYTC	Degenerate RT-PCR
GSP-F1	GACGCTAAGATTGGGAGGGACAGG	5' RACE
GSP-F2	CTGGAAGCCACTATCAGAGGTG	5' RACE
GSP-R1	TAGCAGGACCTCCTTTGCATGGTG	3' RACE
GSP-R2	AATGTCTCCGTATTTCTTCTGCAG	3' RACE
ORF-F	GCTAGCATGGCATGGTTTATTTGTTTG	Cloning
ORF-R	CTCGAGCTAGCCTGACTTAGACTCTTG	Cloning
qRT-F	CCATGGCTCCAGCTCTTTCC	Real-time PCR
qRT-R	CAGTAAGACCAACATCCTGAGTGC	Real-time PCR

Table 1. List of primers used for cloning and expression of *P450c17*.

Functional characterization of catfish *P450c17*

COS-7 cells were grown in DMEM medium supplemented with 10% fetal bovine serum. Cells were transfected using Tfx20 (Promega) with either pcDNA3.1 (Mock) or pcDNA3.1 containing cDNA encoding catfish *P450c17*. After 24 hours, fresh medium was added and 50,000cpm/well of ^{3}H progesterone (Amersham) was added. Cells were then incubated at 37°C and medium was collected at the specified times. Steroids in the medium were extracted twice with 3ml of diethyl ether, separated on thin layer chromatography (TLC) plates using benzene:acetone (4:1 v/v) solvent system and analyzed using phsophorimager. The signals were identified based on the Rf values of standards.

Real-time RT-PCR

Expression of *P450c17* was analyzed by real-time RT-PCR as described in chapter 2 using *P450c17* primer pair listed in table 1. Transcript abundance of *P450c17* was normalized to that of *β-actin* and reported as fold change in abundance relative to the values obtained for spawning phase using the formula $2^{-\Delta\Delta CT}$.

Western blot analysis

Analysis of P450c17 protein levels following hCG-induced oocyte maturation, *in vitro* and *in vivo* as well as in different stages of ovarian cycle was done by Western blot using anti-cfP450c17 polyclonal antiserum.

Enzyme assay

17α-hydroxylase and C17-20 lyase activities of catfish ovarian P450c17 were assayed as described by Zhang *et al*. (1995). Ovarian follicle microsomes were prepared by homogenizing 500 mg of tissue in 3 ml of 0.25 M sucrose/5mM EDTA pH 7.4, clearing debris at 9000Xg for 20 min, and centrifuging at 105,000Xg for 1 hour. The crude microsomal pellet was washed in 0.1 M K. PO_4 pH7.4/0.1 mM EDTA; microsomes were harvested at 105,000Xg for 1 hour, resuspended in 200 µl of 0.1 K. PO_4 buffer/0.1mM EDTA/20% v/v glycerol. P450 activity was measured by incubating 100 µg of microsomal protein and 1 nmol of [^{3}H] progesterone in 0.2 ml of 100 mM K. PO_4 (pH7.4), 1 mM $MgCl_2$, 0.4 mM $NADP^+$, 5 mM glucose-6-phosphate and 0.2 unit of glucose-6-phosphate dehydrogenase at 37°C for 1 hour. Steroids were extracted with three volumes of diethyl ether and analyzed by TLC in benzene/acetone (4:1 v/v) solvent system. Reaction products were identified by Rf values of the respective standards, eluted in alcohol and the radioactivity was measured using liquid scintillation counter.

Data analysis

All the data were expressed as mean ±SEM. Significant among groups was tested by ANOVA followed by Student's-Newmann-Keuls' test using Sigmastat 3.1 software. Differences among groups were considered at $P<0.05$.

Results

Molecular cloning of catfish ovarian *P450c17*

Using a set of degenerate primers, a partial cDNA of 911 bp was isolated by RT-PCR and sequence identity was confirmed by BLAST search. Full-length cDNA of *P450c17* was then obtained through 5' and 3' RACE approaches using gene specific primers designed from partial cDNA fragment (Fig 1). The cloned full length cDNA encoding *P450c17* from the ovary of air-breathing catfish was 2071 bp in length with a 463 bp 3' untranslated region (UTR) and 66 bp 5' UTR. The 3' UTR has one polyadenylation signal. The open reading frame was 1542 bp long encoding a putative enzyme of 514 amino acids (Fig 2). ClustalW multiple alignment demonstrated the presence of signature domains including heme binding region, Ozol's tridecapeptide and Ono sequence were well conserved in catfish *P450c17* and all these signature domains showed high homology to that of *P450c17-I* of other teleosts (Fig. 3). In contrast, catfish *P450c17* exhibited considerable difference in amino acid sequence in Ono sequence and Ozol's tridecapeptide of *P450c17-II* while heme binding region seems to be more or less similar in both forms of *P450c17* (Fig.3). Phylogenetic analysis revealed that catfish *P450c17* has about 60-87% homology with other teleost *P450c17-I*. In contrast it exhibited only about 41-43% homology to that of teleost *P450c17-II* (Fig. 4).

Genomic Southern and Northern blot analyses

Southern analysis of genomic DNA prepared from ovarian follicles identified single band in all the restriction digests when probed with a partial cDNA fragment that possess heme binding region (Fig. 5A). Northern blot analysis identified single transcript of about ~2.1 kb both in testis and ovary (Fig. 5B).

Functional characterization in COS-7 cells

A cDNA corresponding to the open reading frame of catfish *P450c17* was obtained by PCR and cloned in to mammalian expression vector pcDNA3.1. COS-7 cells transfected with *P450c17* expression construct was able to convert progesterone to AD through the intermediate 17α- OHP demonstrating that the cDNA product was indeed functional possessing both hydroxylase and lyase activity (Fig. 6). Similar results were obtained with HEK293 cells after *P450c17* expression (data not shown).

Tissue distribution

Using RT-PCR analysis, *P450c17* expression was detected in several tissues other than gonads including brain, gill, liver, intestine, kidney, heart and muscle. The expression was relatively higher in gonads, brain, kidney and spleen (Fig. 7).

Stage dependent expression of *P450c17* in ovary and testis

P450c17 transcript level found to be high during expressed the preparatory and pre-spawning phases while it was low level in spawning and regressed phases (Fig. 8A). Western blot analysis of protein expression in these phases of ovarian follicles was in accordance with mRNA levels (Fig. 9A). Consistent with expression, the ratio of lyase to hydroxylase activity was high in preparatory and pre-spawning phases while it was low in spawning and regressed phases (Fig. 9B). Cf-P450c17 antiserum characterization details were presented in figures 10 & 11. Real-time RT-PCR analysis demonstrated that the expression of *P450c17* was high in preparatory and spawning phases of testicular cycle while it was low in pre-spawning and regressed phases (Fig. 8B).

P450c17 expression and activity during hCG-induced oocyte maturation, *in vitro* and *in vivo*

There was significant increase in the expression of *P450c17* by two hours after treatment with hCG both *in vitro* and *in vivo* compared to saline-treated controls (Fig. 12). On the contrary, Western blot analysis revealed no changes in protein levels of P450c17 (Fig. 13). Concomitantly, no significant difference was noticed between the ratio of lyase to hydroxylase activity (Fig. 14).

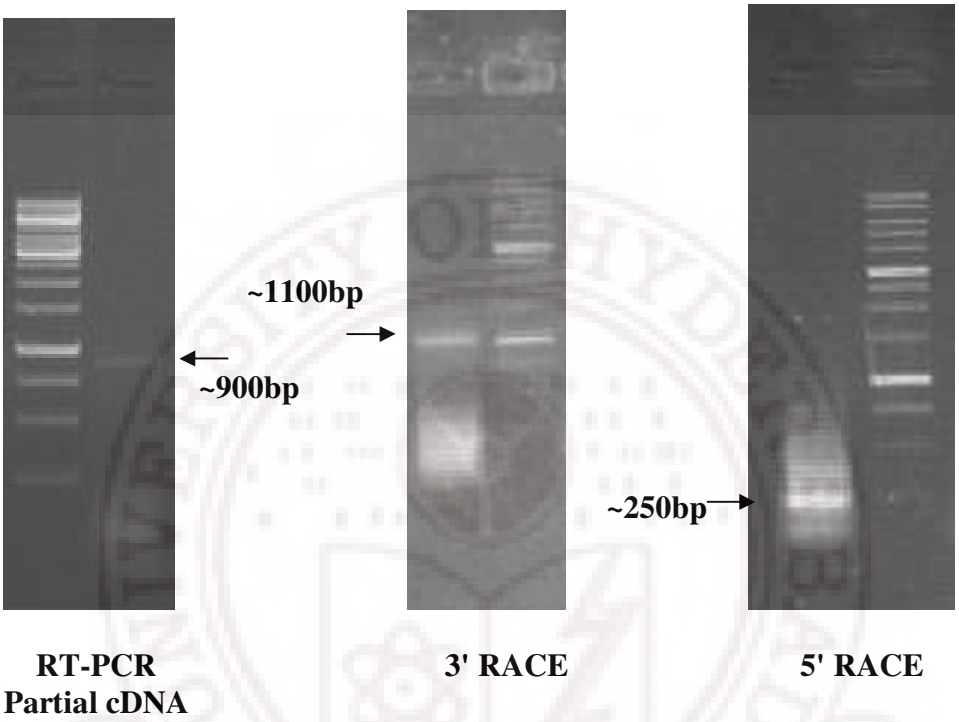

Fig. 1. 1% Agarose gels showing RT-PCR products using degenerate primers for amplification of partial *P450c17* cDNA fragment, 3' and 5' RACE products using gene specific primers to obtain *P450c17* full-length cDNA. Marker in all the gels used was 1 kb ladder.

Fig. 2. Nucleotide (blue) and deduced amino acid (red) sequence of catfish ovarin P450c17. UTRs are shown in black letters and polyadenylation signal is shown in boldface letters with underline.

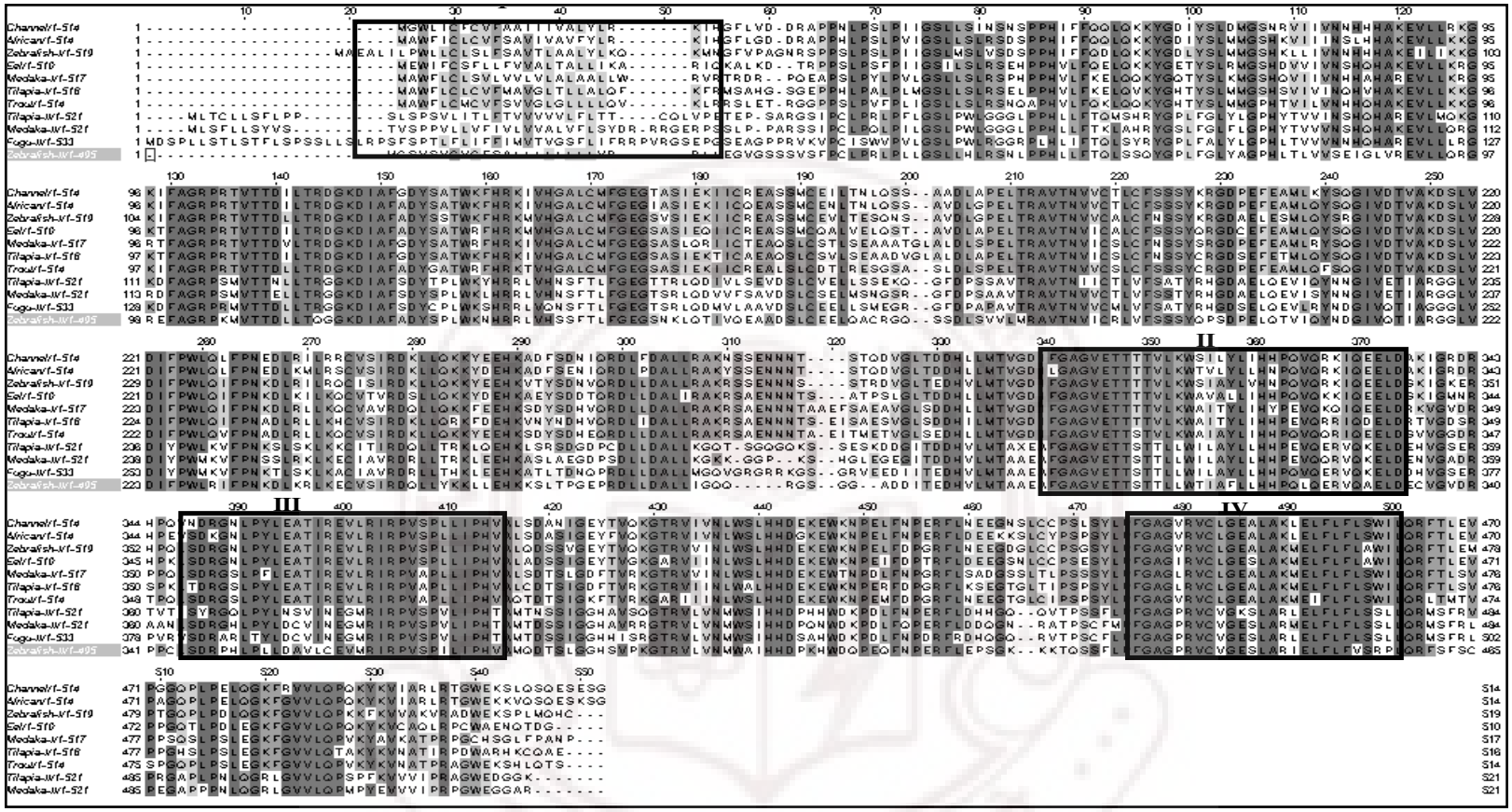

Fig. 3. Alignment of deduced amino acid sequence of catfish P450c17 with those of P450c17-I and P450c17-II of other telesots by ClustalW multiple alignment. Conserved domains are shown in rectangles. I; putative membrane spanning region, II; the P450c17 specific Ono sequence, III; Ozols tridecapeptide regions, IV; the heme binding region. (Accession no.: Trout NM_001124747; Eel AY498619; Tilapia-I AB292401; Tilapia-II EF423917; Zebrafish-I AY281362; Zebrafish-II EF624003; Medaka-I NM_001105094; Medaka-II EF429318).

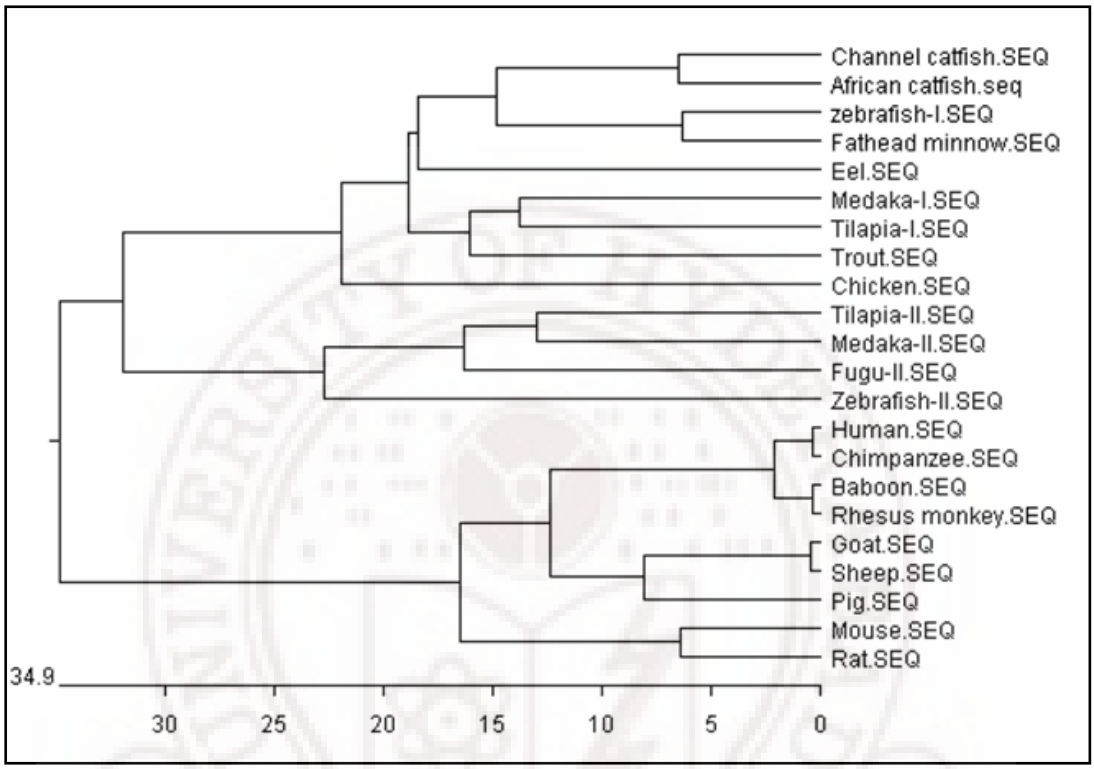

Fig. 4. Phylogenetic tree showing the evolutionary relationship of catfish P450c17. (Please refer fig. 2 for accession numbers of teleost *P450c17s*. Accession no.: Rhesus monkey NM_001040232; Pig NM_214428; Goat AF251387; Chimpanzee NM_001009052; Baboon AF297650; Sheep AF251388; Chicken M21406; Human M14564; Mouse NM_007809; Rat M31861; Frog AF325435).

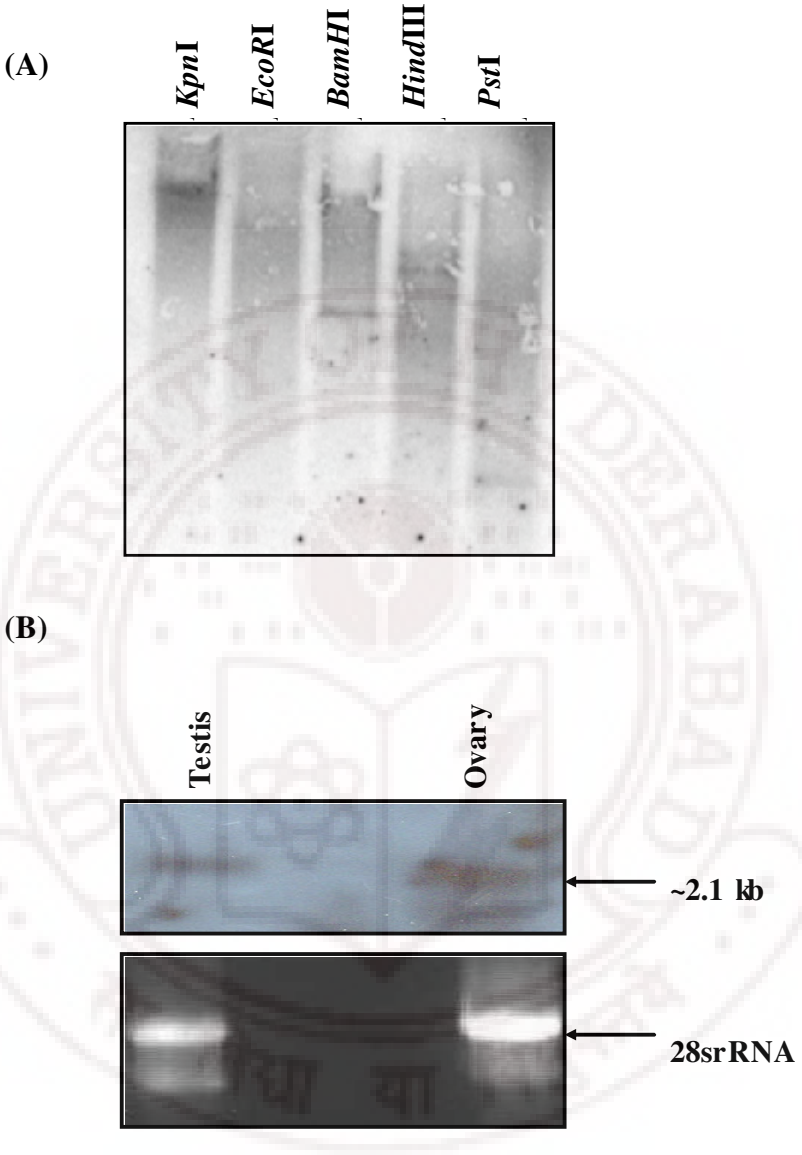

Fig. 5. Genomic Southern analysis of catfish ovarian follicles probed with a partial cDNA lacking sites for the enzymes used in digestion (A). 1kb DNA ladder was used to identify the size of signals. Northern blot analysis of 25 µg of total RNA from catfish ovary and testis (B). RNA ladder was used to identify the size of bands.

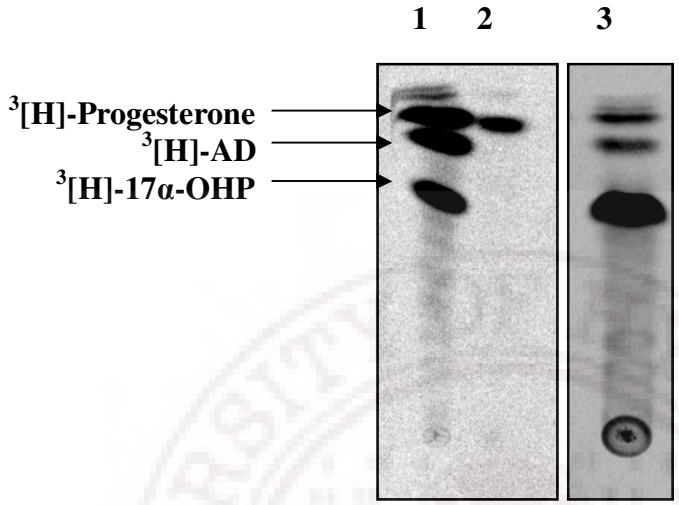

Fig. 6. Autoradiogram showing the 17α-hydroxylase and C17-20 lyase activities of catfish *P450c17* transiently expressed in COS-7 cells. Lanes 1. Standards, 2. pcDNA3.1, 3. pcDNA3.1-*P450c17*.

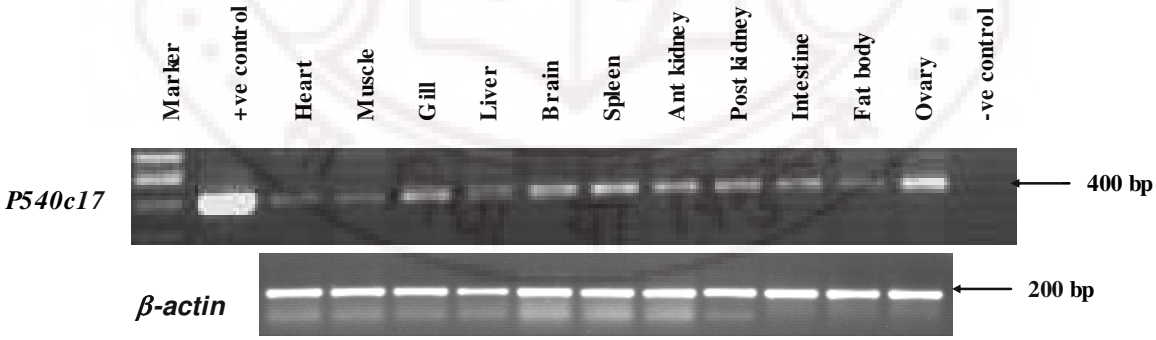

Fig. 7. RT-PCR analysis of spatial expression pattern of catfish *P450c17* in different tissues. Plasmid clone containing catfish *P450c17* was used as positive control (+ve ctl). Negative control (-ve ctl) contains no cDNA template. Ant. kidney, Anterior kidney; Post. kidney, Posterior kidney. Marker used was 100 bp ladder.

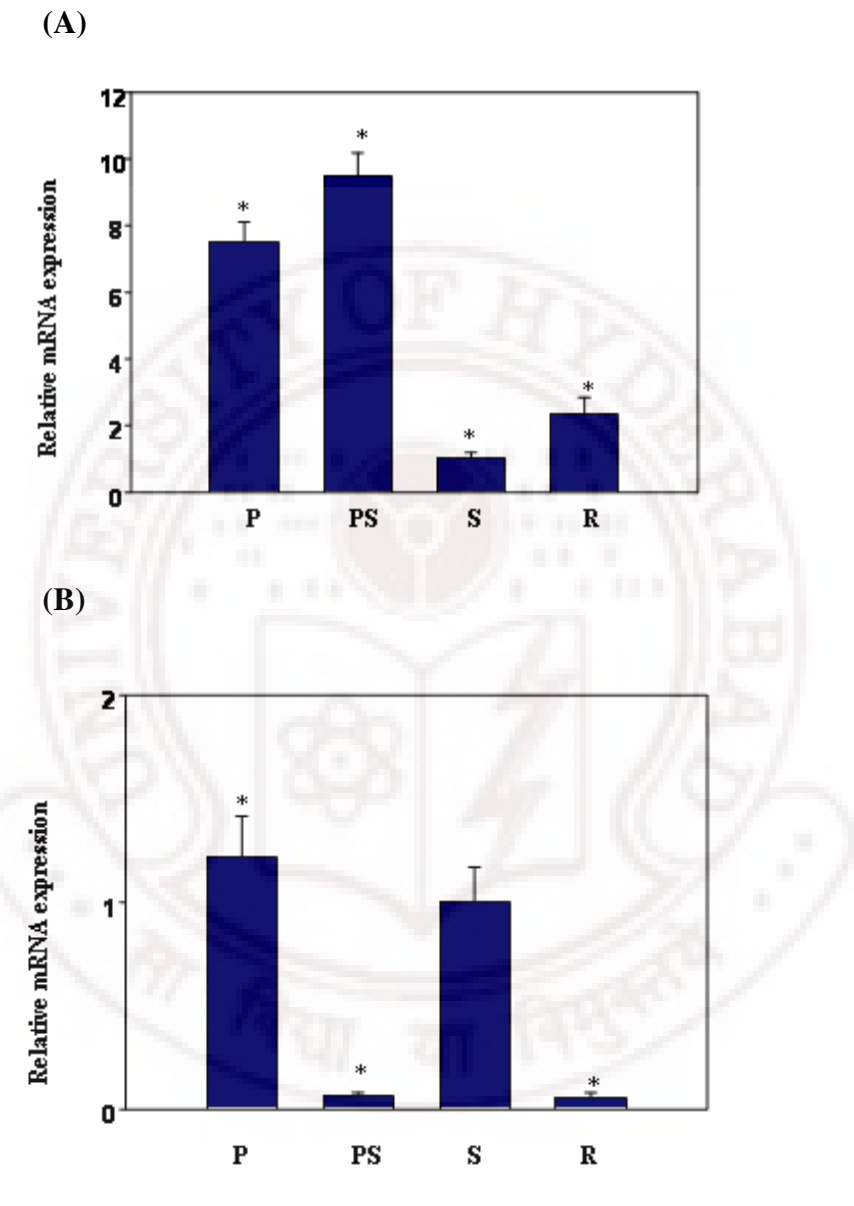

Fig. 8. Real-time RT-PCR analysis of *P450c17* expression in catfish ovarian (A) and testicular (B) cylces. P, Preparatory; PS, pre-spawning; S-spawning; R, regressed (* indicates the significance, n=3, *P*<0.05, ANOVA).

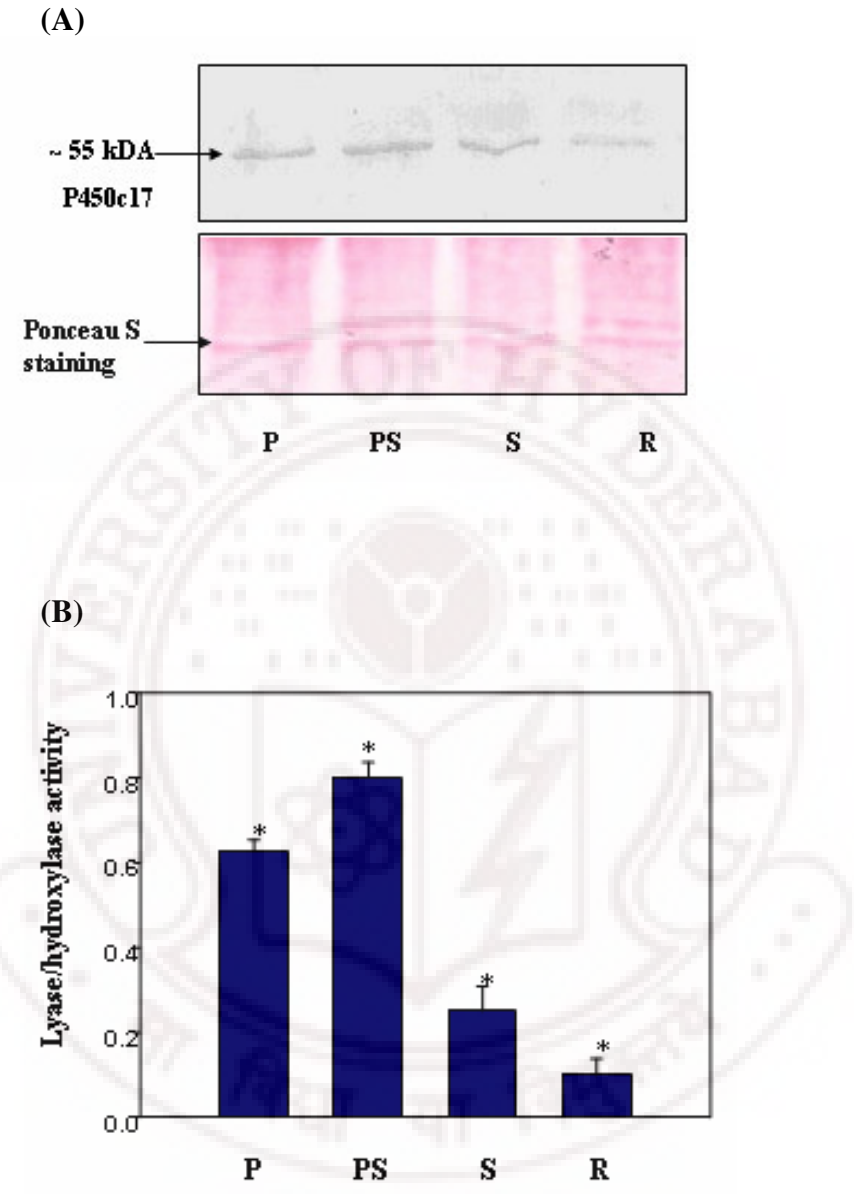

Fig. 9. Representative Western blot showing P450c17 expression in different stages of ovary (A). Ponceau S staining was used to depict equal loading (lower panel). Lyase to hydroxylase ratio in different stages of ovarian cycle (B). P, Preparatory; PS, Pre-spawning; S, Spawning; R, Regressed (* indicates the significance n=3, *P*<0.05, ANOVA).

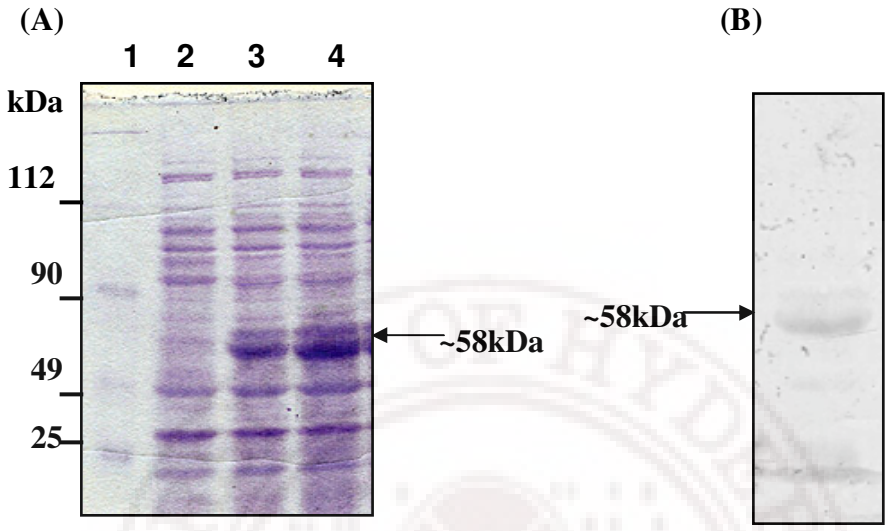

Fig. 10. A 10% SDS-PAGE showing the expression of catfish recombinant *P450c17* in *E. coli* BL21 (A). Lane 1, protein marker, lane 2, un-induced and lanes 3, 4 are induced with IPTG at 2 and 4 hrs respectively. Western blot analysis of recombinant P450c17 with mouse anti-His antibody (B).

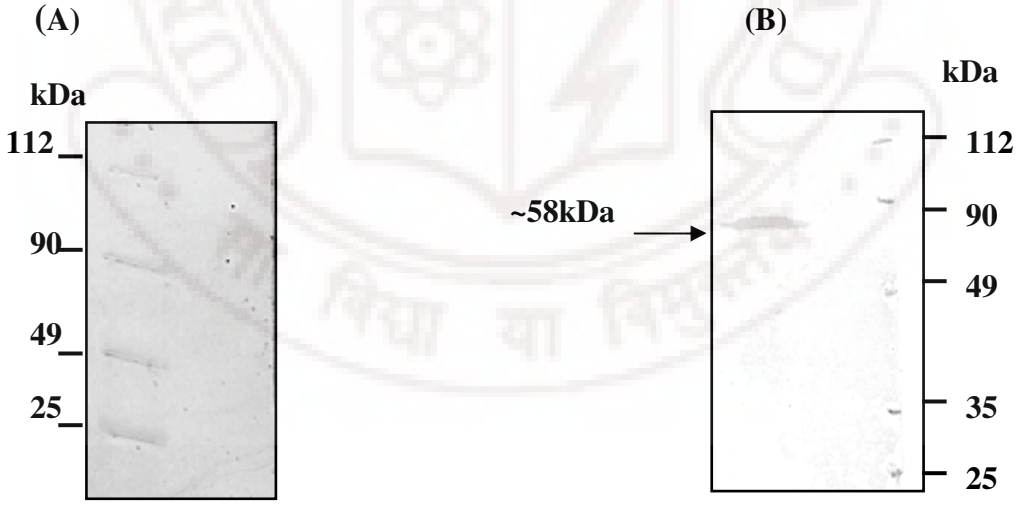

Fig. 11. Western blot analysis of catfish recombinant P450c17 with Pre-immune serum (A) and rabbit anti-cfP450c17 (B).

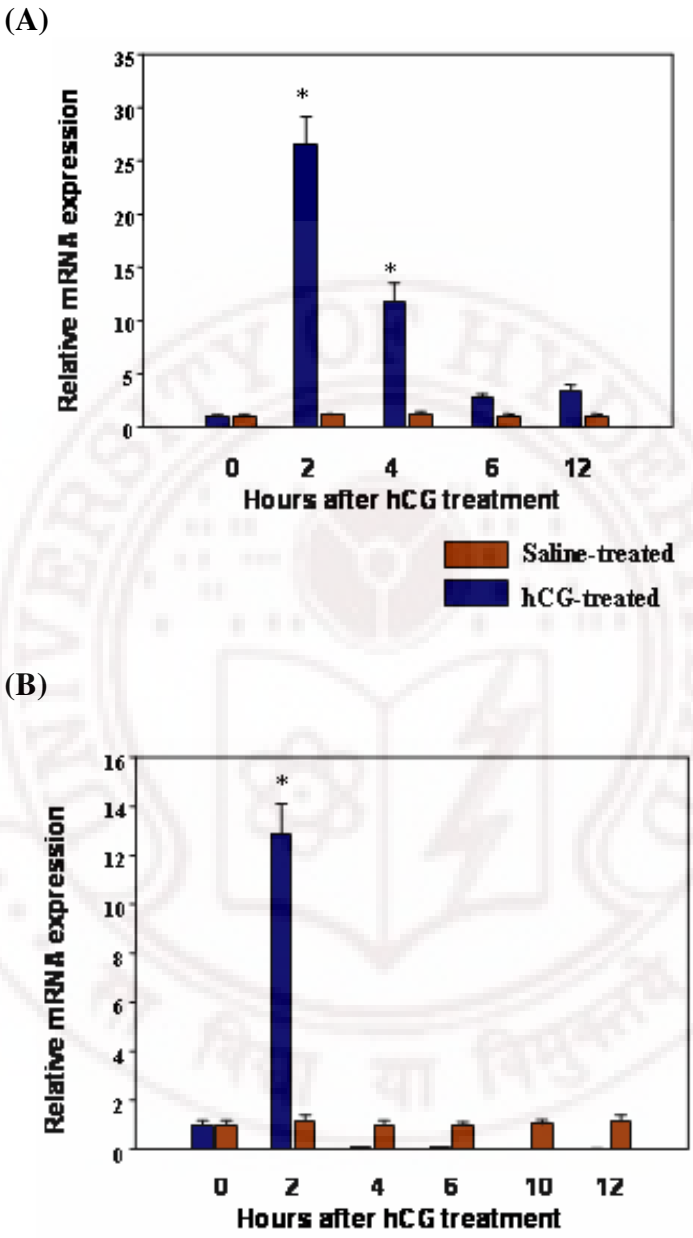

Fig. 12. Real-time RT-PCR analysis of *P450c17* expression during hCG-induced oocyte maturation *in vitro* (A) and *in vivo* (B). * Indicates the significance (n=3, *P*<0.05, ANOVA).

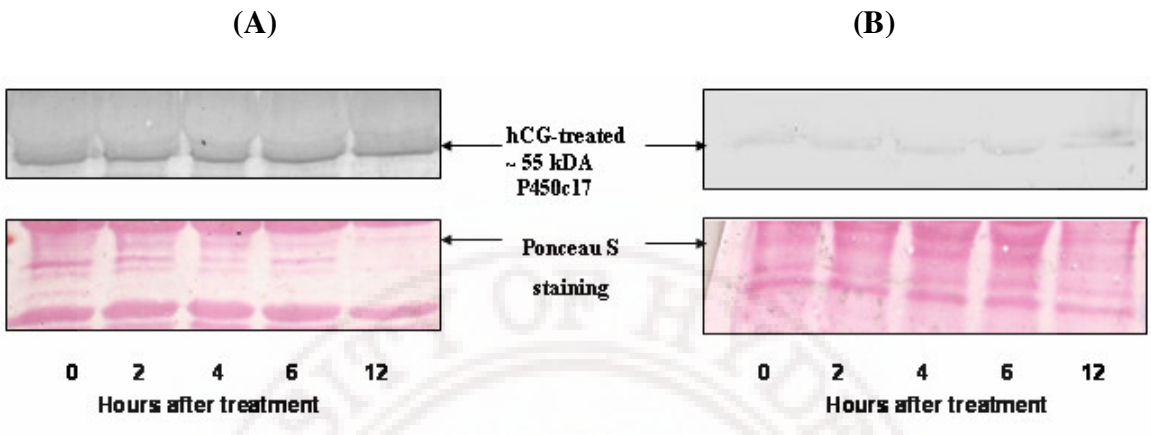

Fig. 13. Western blot analysis (representative, n=3) of P450c17 during hCG-induced oocyte maturation *in vitro* (A) and *in vivo* (B). Ponceau S staining was used to depict equal loading (lower panel).

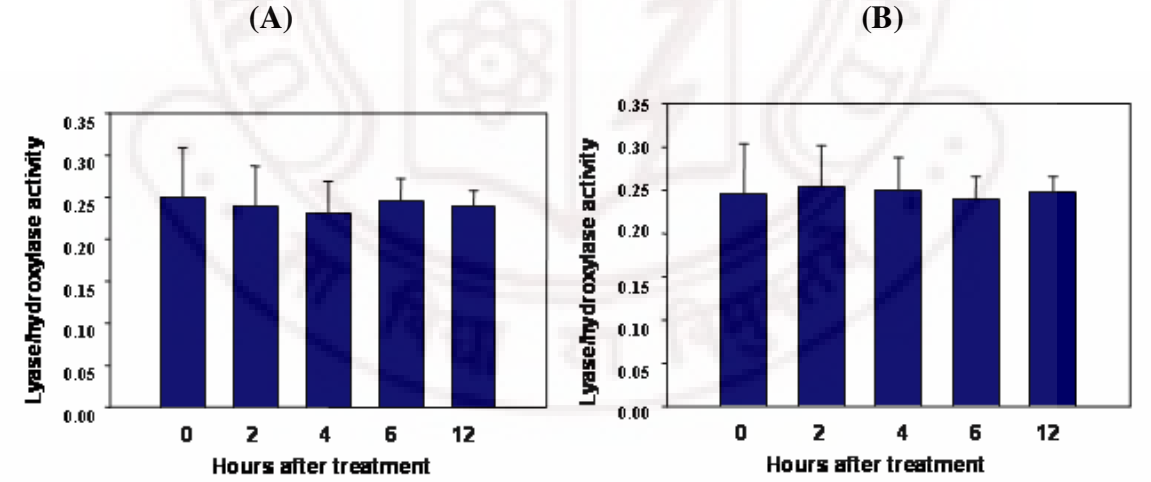

Fig. 14. Changes in lyase to hydroxylase ratio during hCG-induced oocyte maturation *in vitro* (A) and *in vivo* (B).

Discussion

In the present study, we used hCG-induced oocyte maturation both *in vitro* and *in vivo* to delineate the role of *P450c17* in steroidogenic shift. To achieve this, a full-length cDNA encoding *P450c17* was isolated from catfish ovarian follicles. The catfish *P450c17* has high homology to that of *P450c17-I* cDNA sequences reported in other teleosts and their counterparts in higher vertebrates. The close relationship of *P450c17*s among these species is likely to be common. In general, P450 enzymes exist as single gene in multiple species mediating multiple enzymatic steps (Miller, 2002) and multiple forms of *P450c17* have not been found in mammals and other lower vertebrates except in few teleost species in which a second form of *P450c17* that lacks the lyase activity (Zhou *et al*., 2007a & b). However, since we used partial cDNA fragment spanning the common heme binding region as probe in genomic Southern analysis, our identification of single copy gene for *P450c17* seems to be reasonable. COS-7 cells transfected with catfish *P450c17* cDNA converted exogenous progesterone to 17α-OHP and AD that is in accordance with previous reports in which only single form of *P450c17* was identified. Consistent with cDNA cloning and genomic Southern analysis, a single transcript was detected both in ovary and testis.

The observed positive correlation of *P450c17* expression in catfish gonadal cycle shares similarity with other teleosts like trout (Sakai *et al*., 1992), eel (Kazeto *et al*., 2000) and channel catfish (Kumar *et al*., 2000). However, there was no obvious association of *P450c17* expression with ovarian development in fathead minnow (Halm *et al*., 2003)

while the expression of *P450c17* was high throughout all the developmental stages of ovarian follicles without any significant difference among the developmental stages in zebrafish (Wang and Ge, 2004). The discrepancy in expression pattern of *P450c17* among these species could be attributed to the sensitivity of the techniques used or the varied pattern of reproductive cycles of zebrafish and fathead minnow compared to eel, trout and catfish. On the other hand, in tilapia *P450c17-I* expression peaks about midvitellogenic stage and thereby decreases with maturational stage while *P450c17-II* expression was maximum during maturation. Consistent with mRNA, protein levels and ratio of lyase to hydroxylase activity in different stages of follicle development (present study) corroborates to *P450c17* expression in eel and channel catfish (Kazeto *et al*., 2000; Kumar *et al*., 2000). To our knowledge, present study was first of its kind to correlate expression analysis to enzyme activity for *P450c17*. The higher expression of *P450c17* during early and mid stages of follicle development is presumed to be important for production of higher levels of Δ^4 steroids and is supported by the correlation between expression and enzyme activity (Kumar *et al*., 2000). Intriguingly, a negative correlation was identified with testicular development in fathead minnow (Halm *et al*., 2003) while we found a maximum expression of *P450c17* in preparatory and spawning phases of catfish testis. The expression pattern of *P450c17* in catfish testis matches with levels of Δ^4 steroids in preparatory and 17α, 20β-DP levels in spawning phase.

Consistent with previous reports in mammals, birds and fishes, we could also detect *P450c17* transcript in many tissues of catfish. *P450c17* was originally thought to be present exclusively in gonads and adrenals, later on mRNA, protein and/or activity were found in several other tissues including the brain (Yu *et al*., 2002; Halm *et al*., 2003;), gastrointestinal tract, (Dalla Valle *et al*., 1995) and liver (Katagiri *et al*., 1998). In agreement with its widespread localization, *P450c17* is a potent oxidant and catalytic reactions other than hydroxylation and lyase have been suggested (Lieberman and Warne, 2001). Ying Liu *et al*. (2005) reported that *P450c17* also functions as squalene monooxygenase involved in cholesterol biosynthesis.

The 17α-hydroxylase activity of P450c17 is necessary for synthesis of cortisol in adrenal while the lyase activity becomes important for production of sex steroids in gonads (Payne and Hales, 2004). The mechanism behind organ-specific differential actions of P450c17 was attributed to post-translational modification regulations such as abundance of electron donating partner P450 oxidoreductase, cytochrome b5 and Ser/Thr phosphorylation in mammals (Zhang *et al*., 1995). Alternatively, the presence of P450c17 isoenzymes has been proposed to explain differential actions of P450c17. In teleost ovary, lyase activity is required for the production of estrogens during growth phase while hydroxylase activity is necessary for the production of 17α, 20β-DP during maturational phase (Nagahama, 1997). Until recently (Zhou *et al*., 2007a & b), only one form of *P450c17* that is highly homologous to mammals has been found in few fish species and it has been thought that the similar mechanism found in mammalian

adrenals were responsible for these differential actions of P450c17 (Kazeto *et al*., 2000; Wang and Ge, 2004). In the present study neither protein levels nor enzyme activity showed significant changes to hCG both *in vitro* and *in vivo* although mRNA levels increased. The significance of *P450c17* mRNA rise after treatment with hCG is not clear at present. But the increase in P450c17 mRNA levels could be attributed to the increase in intracellular cAMP levels with hCG as observed in rat Leydig cells (Payne, 1990). It is also plausible that the increase in *P450c17* transcripts during hCG-induced oocyte maturation might have higher input to steady levels of P450c17 protein. This contention needs further evaluation. Similarly in eel, *P450c17* mRNA levels increased gradually through out the artificial induction of gonadal development (Kazeto *et al*., 2000). In contrast, the cultured zebrafish ovarian follicles did not respond to either hCG or activin (Wang and Ge, 2004). In the case of tilapia and medaka, differential expression patterns of two forms of *P450c17* are presumed to be important during FOM (Zhou *et al*., 2007a & b). All the previous reports, including the recent identification of *P450c17-II* (Zhou *et al*., 2007a & b), did not study enzyme activity and regulation of lyase activity in detail. Based on the reports in mammals (Miller *et al*., 1997), it seems to be compulsory to gain knowledge both at the level of mRNA as well as protein and enzyme activity owing to the complexity of regulation of these enzymes. However, this second form of *P450c17* is unique to only two teleost species and it is hypothesized that this gene might have been evolved from the fish specific genome duplication (Hoegg *et al*., 2004; Mayer and Van de Peer, 2005). Hence differential actions of P450c17 with

presence of multiple forms can not be generalized to all the teleost species. Though present study categorically demonstrated the *P450c17* expression and activity during oocyte maturation, regulation of lyase activity in telesots possessing single form of *P450c17* continues to be an issue which needs to be resolved. Further analysis assessing the regulation of lyase activity with reference to P450 oxidoreductase and cytochrome b5 is of worthy that might provide interesting clues about the regulation of P450c17 in telesots possessing single form of *P450c17*.

In conclusion, a single form of *P450c17* that is homologous to *P450c17-I* has been cloned from the ovarian follicles of catfish. *P450c17* mRNA, protein levels and activity was found to be high during preparatory and pre-spawning stages of follicle development while testis has maximum expression during preparatory and spawning stages. During hCG-induced oocyte maturation both *in vitro* and *in vivo*, neither protein level nor ratio of lyase/hydroxylase changed significantly although there was an increase in mRNA levels by 2 hr after induction with hCG. Taken together, it seems that *P450c17* potentiates during preparatory/pre-spawning phases which might exert influence on the shift in steroidogenesis during ovarian and testicular recrudescence.

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Abstract

Complementary DNAs encoding steroidogenic acute regulatory protein (*StAR*) have been isolated from different fish species, yet the relevance of *StAR* during gonadal cycle and more importantly in final oocyte maturation has not been assessed so far. A cDNA encoding *StAR* was isolated from the ovarian follicles of air-breathing catfish, *Clarias gariepinus*. Catfish *StAR* exhibited 55 to 72% identity at nucleotide level with other vertebrate orthologs. RT-PCR analysis of tissue distribution pattern demonstrated the presence of *StAR* mRNA in various tissues including gonads, head kidney, liver, brain and intestine of catfish. Real-time RT-PCR analysis revealed high expression of *StAR* mRNA in the pre-spawning phase of ovary while it was low in preparatory, spawning and regressed phases. In testis, maximum expression was noticed during the preparatory phase. During human chorionic gonadotropin (hCG)-induced oocyte maturation, both *in vitro* and *in vivo*, *StAR* mRNA levels were augmented by 2 hrs and then declined gradually to reach basal levels by 12 hrs as that of saline-treated controls. Taken together, high level of expression during hCG-induced oocyte maturation vis-à-vis in spawning suggests a role for *StAR*, in addition to the steroidogenic enzyme genes in final oocyte maturation.

Introduction

Steroid hormones play a crucial role in the regulation of growth, development, differentiation, reproduction and several other functions in vertebrates. Production of different classes of steroids occurs from a common precursor, cholesterol and involves a battery of oxidative enzymes (Payne and Hales, 2004). The first committed step in steroid hormone biosynthesis is the conversion of cholesterol to pregnenolone, which occurs in mitochondria by the action of P450 side chain cleavage enzyme (P450scc). However, cholesterol cannot cross the mitochondria from cytoplasm and is delivered by a sterol transfer protein, steroidogenic acute regulatory protein (*StAR*; Stocco, 2000). Now it has been accepted that the true rate limiting step in steroidogenesis is the delivery of cholesterol across mitochondrial membrane. This is an important target for acute steroidogenesis by tropic hormones (Stocco, 2001), few other mediators and some endocrine disruptors (Walsh and Stocco, 2000) as well. Therefore, *StAR* is indispensable for mediating cholesterol transfer vis-à-vis steroidogenesis. Perhaps the most compelling evidence arose from the identification of mutations in *StAR* gene during congenital adrenal hyperplasia (CAH), a condition in which cholesterol and cholesterol esters accumulate and the newborn is unable to synthesize adequate levels of steroid hormones (Lin *et al*., 1995). This is further evidenced by *StAR* knockout mice which showed phenotypic mirrors of human lipoid CAH (Caron *et al*., 1997).

A cDNA encoding a 30-kDa mouse StAR was first characterized by Clark *et al*. (1994). StAR is believed to transfer cholesterol across mitochondria either by forming a

transport tunnel (Tsujishitay and Hurley, 2000) or by a cavity (Mathieu *et al*., 2003). It is plausible that StAR interacts with contact sites where the inner and outer mitochondrial membranes are in close proximity (Thomson, 2003). Further, studies have shown that StAR is rapidly synthesized in response to stimulation of several hormones such as luteinizing hormone (LH), adrenocorticotropic hormone (ACTH; Clark *et al*., 1995) typically with activation of the cAMP second messenger system. Although StAR appears to be critical for steroidogenesis in the adrenal and gonads, some of the tissues that do not express *StAR*, including placenta, synthesize large amounts of pregnenolone suggesting for the existence of StAR-independent mechanisms for movement of cholesterol to P450scc enzyme (Stocco, 2001).

Complementary DNA-encoding proteins with high homology to *StAR* of mammals were cloned from zebrafish, rainbow trout, eel, cod and stingray (Bauer *et al*., 2000; Kusakabe *et al*., 2002; Li *et al*., 2003; Goetz *et al*., 2004, Nunez *et al*., 2005). In teleosts, besides correlating increase in *StAR* mRNA to acute interrenal or gonadal steroid production, very little is know about the dynamics of *StAR* transcripts in relation to gonadal cycle and more importantly final oocyte maturation. A shift in steroidogenesis from estradiol-17β (E_2) to 17α, 20β-dihdroxy-4-pregnen-3-one (17α, 20β-DP) is important for final oocyte maturation that is associated with pre-ovulatory LH surge (Nagahma, 1997; Senthilkumaran *et al*., 2004; Nagahama and Yamashita, 2008). This steroidogenic shift is governed by the down regulation of ovarian P450 aromatase and up-regulation of 20β-hydroxysteroid dehydrogenase, the enzymes that

produce E_2 and 17α, 20β-DP, respectively (Yoshiura *et al*., 2003; Senthilkumaran *et al*., 2004; chapter 1 & 2). However, involvement of *StAR* during shift in steroidogenesis would be possible, owing to the reason that *StAR* is rapidly synthesized in response to trophic hormone stimulation. In the present study, a cDNA encoding *StAR* was isolated from ovarian follicles of the air-breathing catfish, *Clarias gariepinus*. We then analyzed *StAR* transcript abundance during human chorionic gonadotropin (hCG)-induced oocyte maturation, *in vitro* and *in vivo* by real-time RT-PCR. To complement our results, expression of *StAR* was also analyzed during different stages of gonadal cycle.

Materials and Methods

Animals and treatments

Adult catfish of about 400-500g were purchased live from local fish markets (Hyderabad, India) and maintained as described in chapter 2. For seasonal cycle analysis, gonads at different phases of reproductive cycle were collected, snap frozen in liquid nitrogen and stored at -80°C until use. Procedure followed for *in vitro* and *in vivo* oocyte maturation was described in detail in chapter 2.

Cloning of catfish *StAR* cDNA

A set of degenerate primers (listed in table 1) designed by aligning existing fish *StAR* cDNA sequences and partial cDNA encoding catfish *StAR* was cloned from ovary following the method described in chapter 2. Subsequently 5' and 3' rapid amplification of cDNA ends (RACE) was performed using gene specific primers designed from partial cDNA fragment (table 1). Methodology followed for RACE was described in chapter 2. Finally, the open reading frame (ORF) was amplified using gene specific primers and the sequences were confirmed bi-directionally. Phylogenetic analysis was performed with lasergene software (DNASTAR,version 3) by neighbor-joining method.

Primer	Sequence 5' – 3'	Purpose
DF1	TGTGHGCTGGCATHTCHTAC	Degenerate PCR
DR1	GGTGRTTKRCRAARTCCACCT	Degenerate PCR
GSP-R1	CCAACATCCTAGCATGCCTGAGCA	5' RACE
GSP-R2	GACCAAGTTCACCTGGTTACTCAG	5' RACE
GSP-F1	GGATAGCTTGCTCAGCTCGTGGTG	3' RACE
GSP-F2	ATGGCCATCATGGCGTTCCTCCTC	3' RACE
ORF-F	GCTAGCATGCTACCTGCAACTTTTAAG	Tissue distribution
ORF-R	CTCGAGGCAGGCCATTGCCTCCTCCA	Tissue distribution
qRT-F	TGGCCATCCACCACGAGCTG	Real-time PCR
qRT-R	CAATCTCAGTTTGCCAGCCATC	Real-time PCR

Table 1. List of primers used for cloning and expression of *StAR*.

Northern Blot Analysis

Twenty five µg of total RNA from ovary and testis were probed with catfish *StAR* ORF labeled with ^{32}P-dCTP following the steps mentioned in chapter 2. Northern blot analysis was repeated thrice independently.

RT-PCR analysis of tissue distribution

To analyze tissue distribution pattern of *StAR* in catfish tissues, 1 µg of total RNA obtained from different tissues was reverse transcribed and RT-PCR was performed as mentioned in chapter 2 using catfish *StAR* ORF primers.

Real-time RT-PCR

Expression of *StAR* was analyzed by real-time RT-PCR as described in chapter 2 using *StAR* primer pair listed in table 1. Transcript abundance of *StAR* was normalized to that of *β-actin* and reported as fold change in abundance relative to the values obtained for spawning phase using the formula $2^{-\Delta\Delta CT}$.

Statistical analysis

All the real-time RT-PCR data was expressed as ±SEM. Significance between groups was tested by ANOVA followed Student's-Newman-Keuls' test using Sigmastat3.1 software. Differences among groups were considered significant at $P<0.05$.

Results

Cloning of catfish ovarian *StAR*

Using a degenerate primer pair, a partial cDNA of 550 bp was amplified by RT-PCR and sequence was determined, subsequently sequence identity was confirmed by BLAST analysis. Further, full-length cDNA was obtained by 5' and 3' RACE approaches (Fig. 1). Catfish ovarian *StAR* cDNA was 1.150 kb long with an ORF of 858 bp encoding a putative protein of 285 amino acids. 5' Untranslated region (UTR) was 64 bp while 3' UTR was 248 bp long with a potential polyadenylation signal (Fig. 2).

ClustalW multiple alignment of deduced amino acid sequence of catfish *StAR* with other vertebrate orthologs demonstrated that the signature domains such as, mitochondrial targeting sequence, cholesterol recognition site and protein kinase A phosphorylation site are highly conserved among vertebrates (Fig. 3). Phylogenetic analysis revealed that catfish ovarian *StAR* is highly homologous to its fish counterparts with 55-72% identity at nucleotide level where as it exhibited only 55-60% with higher vertebrates (Fig. 4).

Transcript size and sites of expression

Northern blot analysis identified a single band of 1.2 kb in ovary where as in testis a larger transcript of 2.5 kb was also detected in addition to 1.2 kb transcript (Fig. 5). RT-PCR analysis of tissue distribution pattern of *StAR* showed predominant expression

in gonads and liver. In addition, transcript was also found in kidney, brain and intestine (Fig. 6).

Expression in different stages of gonads

Real-time RT-PCR analysis of *StAR* mRNA abundance at different stages of gonadal cycle demonstrated a high expression in pre-spawning phase of ovary while it was low at preparatory, spawning and regressed phases (Fig. 7A). On the other hand, maximum expression was noticed in preparatory phase of testis. *StAR* expression was minimal during pre-spawning and regressed phases of testis (Fig. 7B).

Expression during hCG-induced oocyte maturation, *in vitro* and *in vivo*

In hCG-induced oocyte maturation, *in vitro StAR* mRNA expression showed a remarkable increase by 2hrs followed by a gradual decrease (Fig. 8A). A similar trend was also observed in hCG-induced oocyte maturation, *in vivo* (Fig. 8B).

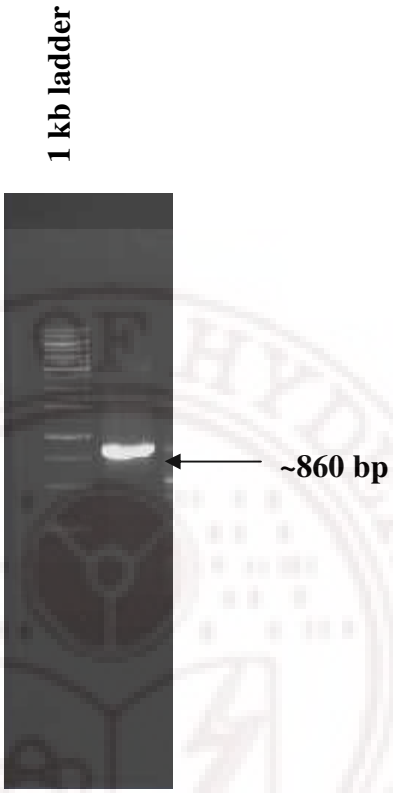


Fig. 1. A 1% agarose gel showing the PCR amplification of catfish ovarian *StAR* ORF.

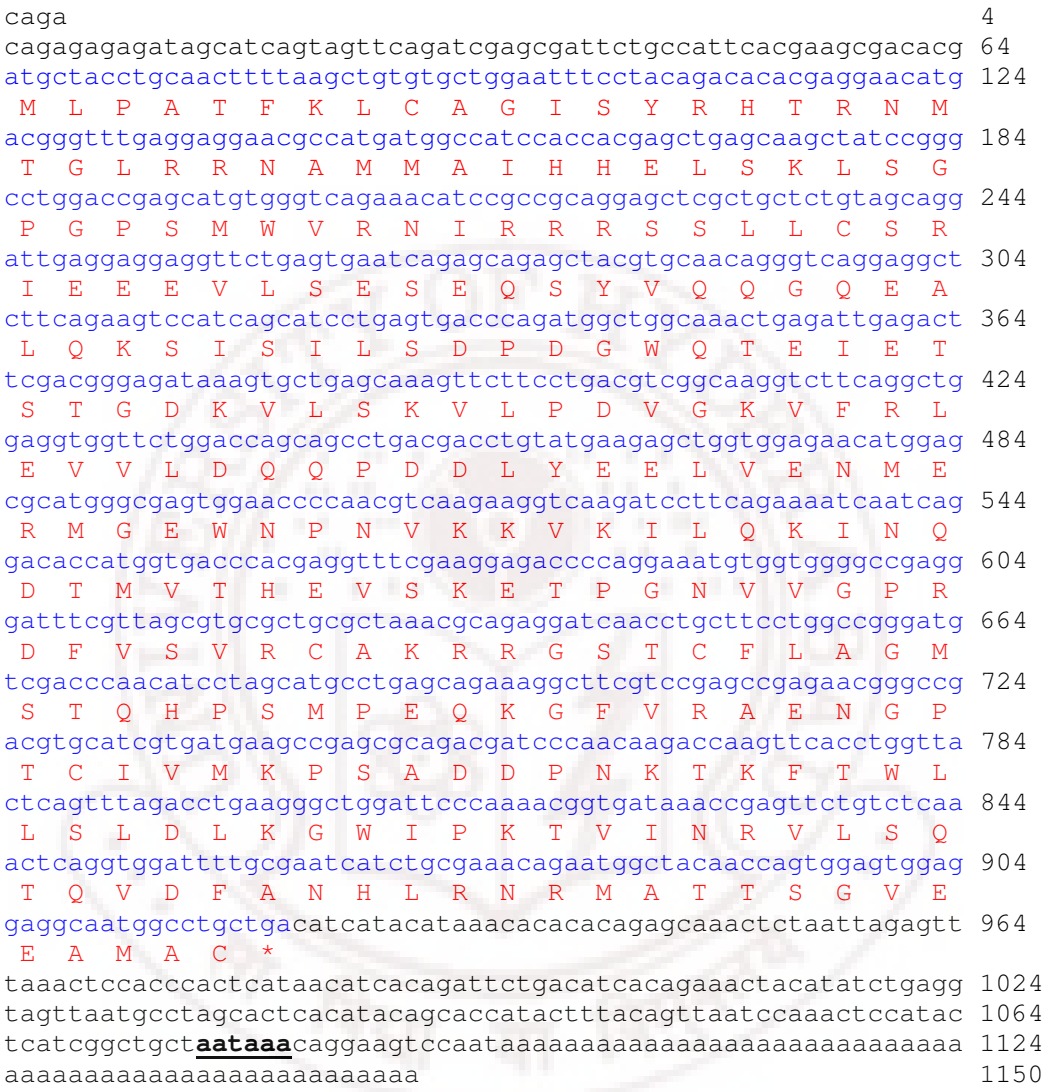


Fig. 2. Nuleotide (blue) and deduced amino acid (red) sequence of catfish ovarian *StAR*. UTRs are shown in black letters. Polyadenylation signal is in boldface letters with underline.

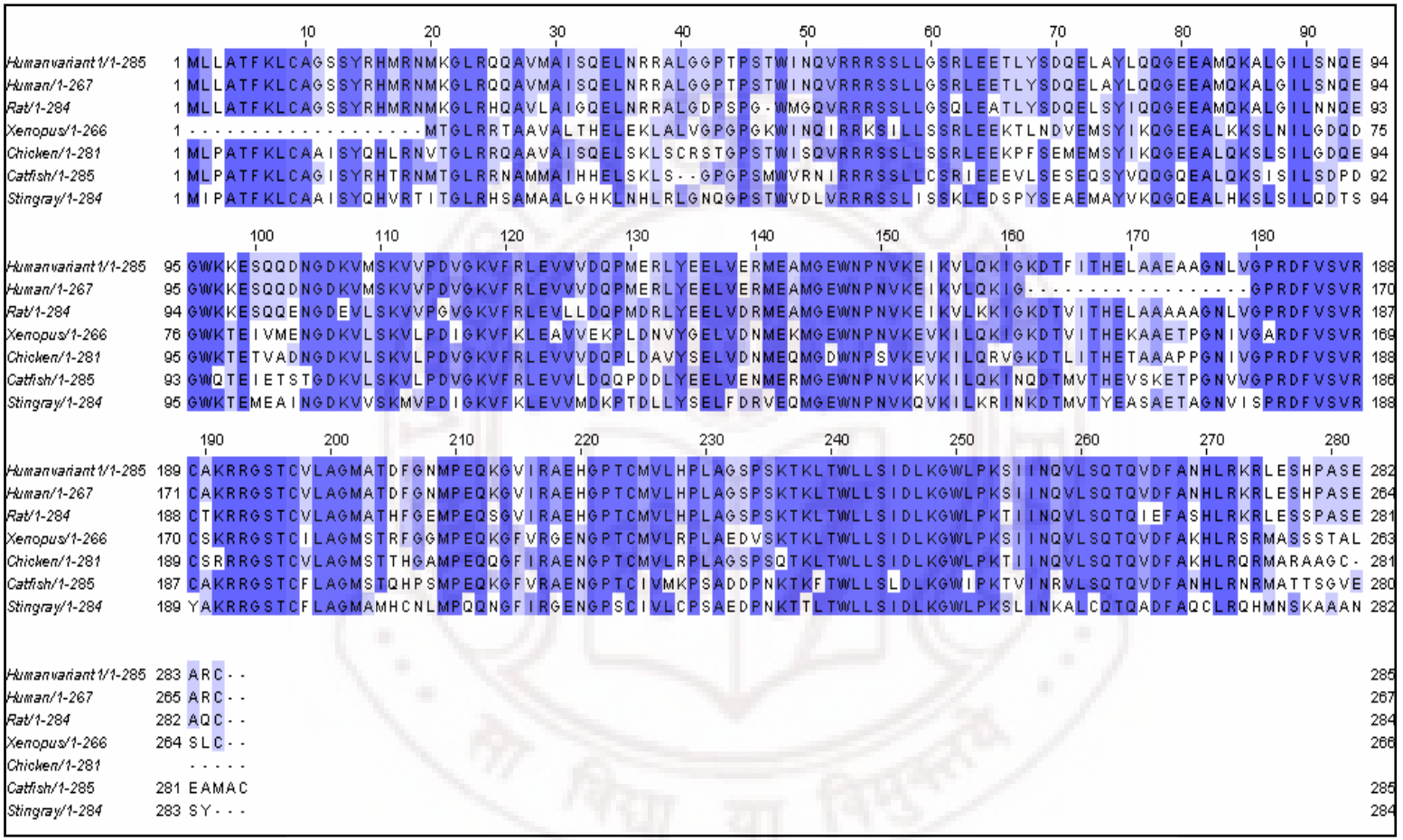


Fig. 3. ClustalW multiple alignment of catfish ovarian StAR with other vertebrate orthologs. (please refere fig. 4 for accession numbers).

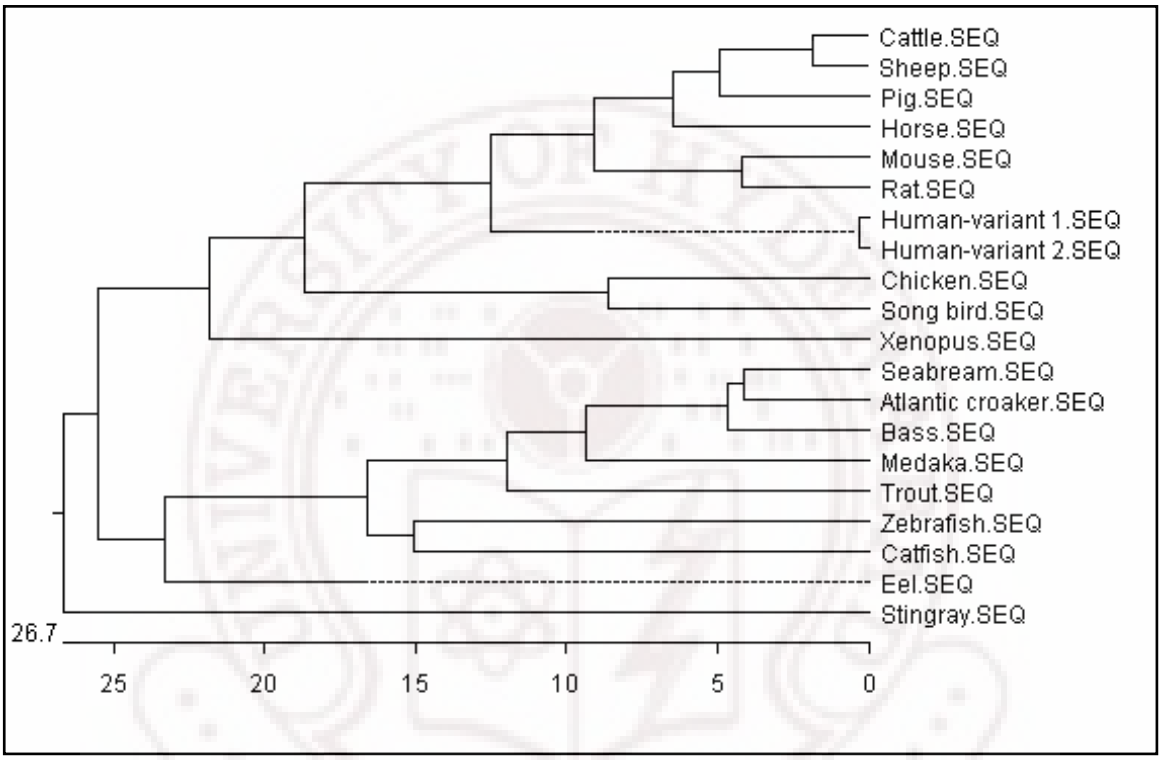


Fig. 4. Phylogenetic analysis of catfish *StAR* showing evolutionary relationship. (Accession No: Human variant 1, **NM_000349**; Human variant 2, **NM_001007243**; Rat, **NM_031558**; Mouse, **NM_011485**; Pig, **NM_213755**; Sheep, **NM_001009243**; Cattle, **NM_174189**, Horse, **NM_001081800**; Song bird, **NM_001076686**; Chicken, **NM_204686**; Xenopus, **AF220437**; Trout, **NM_001124202**; Zebrafish, **NM_131663**; Seabream, **EF640987**; Medaka, **NM_001104910**; Bass, **DQ166820**; Atlantic croaker, **DQ646787**; Eel, **AB095110**; Stingray, **AY553723**).

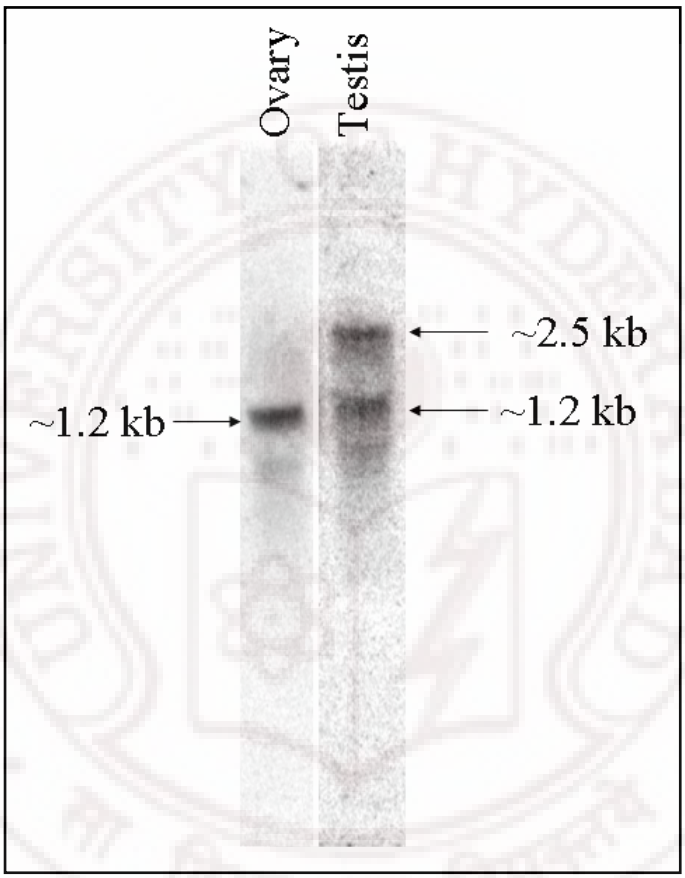


Fig. 5. Northern blot analysis of 25µg catfish ovary and testis total RNA probed with *StAR* ORF. 1 kb RNA ladder was used to track the size of transcript.

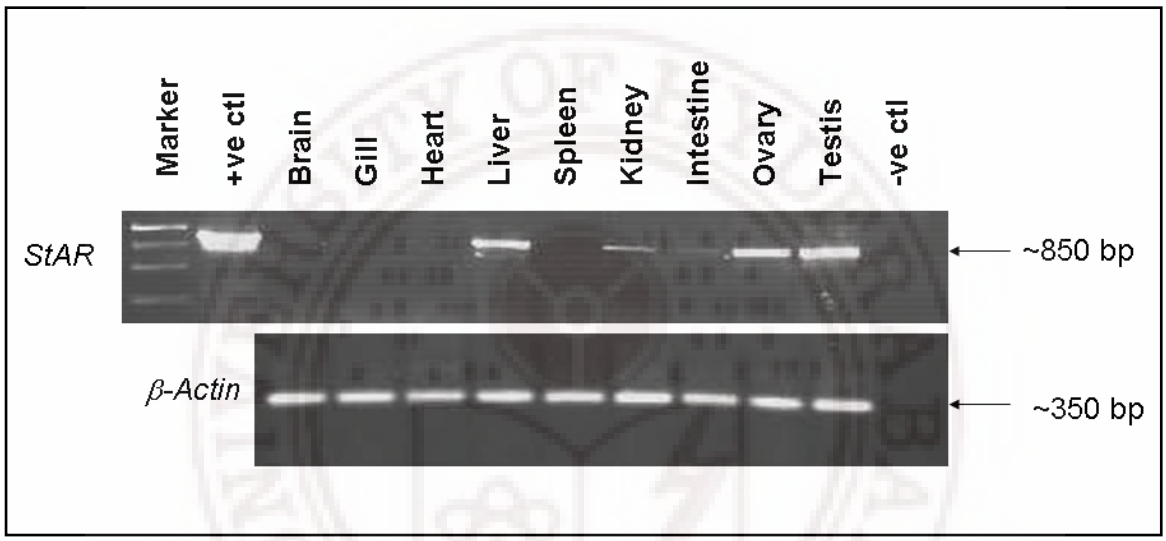
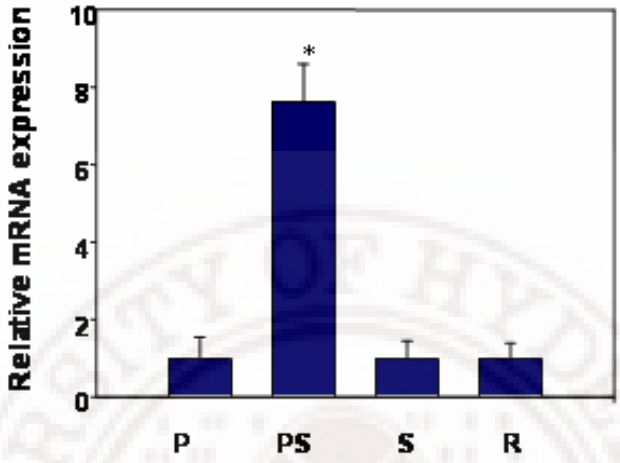


Fig. 6. Spatial expression pattern of *StAR* (upper panel) in various tissues of catfish. *β-Actin* (internal control) is shown in lower panel. Plasmid clone of catfish *StAR* was used as positive control (+ve ctl) and negative control (-ve ctl) contains no cDNA template. Marker used was 1 kb ladder.

(A)



(B)

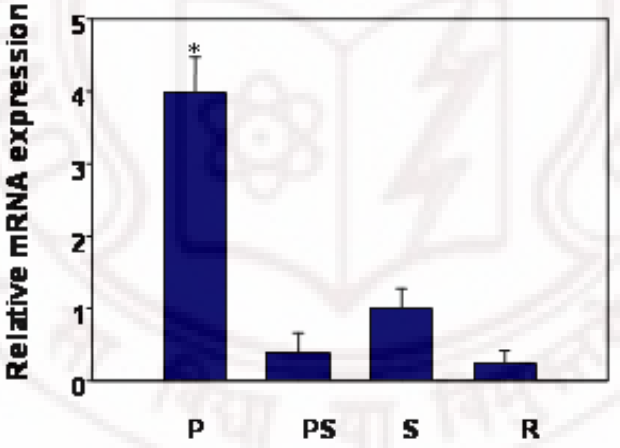


Fig. 7. Stage dependent expression of *StAR* in catfish ovary (A) and testis (B) by real-time RT-PCR. *StAR* expression is reported as fold change relative to spawning phase (* indicates significance, ANOVA, n=3, *P* <0.05). P, preparatory; PS, pre-spawning; S, spawning; R, regressed.

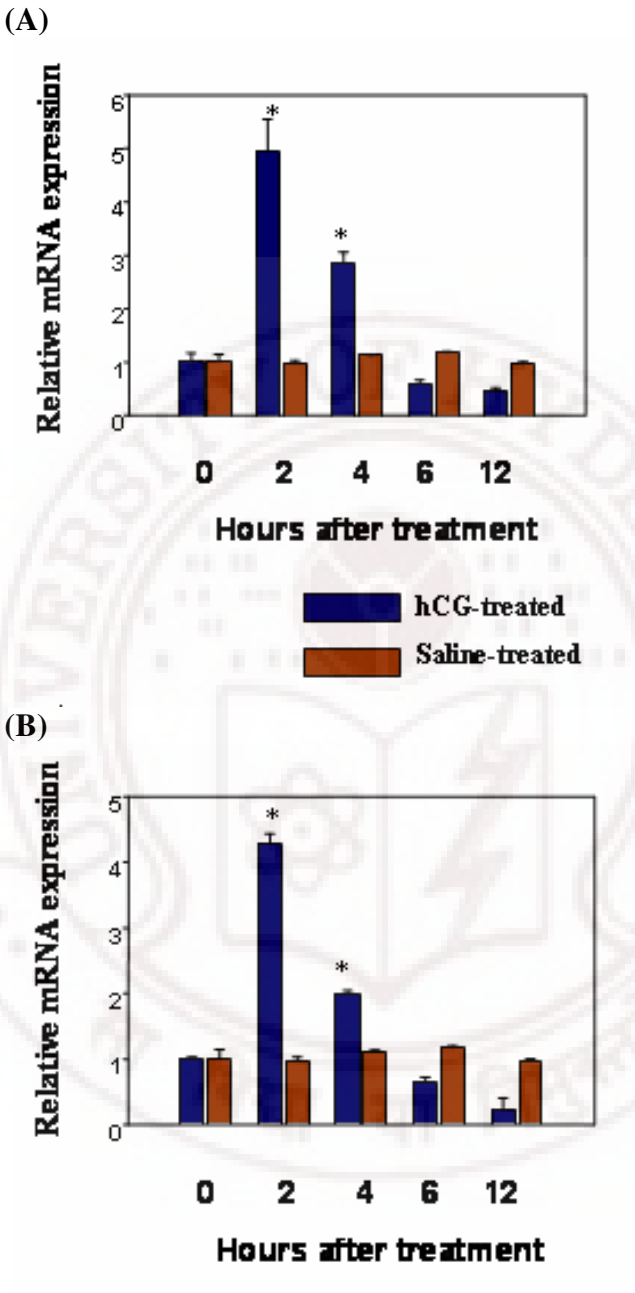


Fig. 8. Real-time RT-PCR analysis showing the expression of *StAR* during hCG-induced oocyte maturation, *in vitro* (A) and *in vivo* (B). *StAR* expression is reported as fold change relative to 0 hrs (∗ indicates significance, ANOVA, n=3, *P* <0.05).

Discussion

Primary goal of this study is to delineate the role of *StAR* during final oocyte maturation. In addition to this, we also assessed changes in *StAR* transcripts during gonadal cycle. Since *StAR* is highly conserved among vertebrates (Bauer *et al*., 2000), we could have directly proceeded for gene expression analysis following hCG-treatment, but presence of multiple forms (including splice variants and UTR variants are known) necessitates isolation of *StAR* cDNA from catfish. Therefore, we cloned a *StAR* cDNA from ovary of catfish. The catfish *StAR* cDNA encodes a protein of 285 amino acids that is similar in size with other teleosts and higher vertebrate *StAR*s identified so far. Phosphorylation of StAR at serine195 is shown to increase the steroidogenic activity (Arakane *et al*., 1997) and in comparison with StARs of other vertebrates, the putative protein phosphorylation site is also conserved in catfish StAR. It is synthesized in cytosol and targeted to mitochondria (Stocco, 2000). The N-terminal region of catfish StAR, composed of basic and hydrophobic amino acids, has characteristic mitochondrial targeting sequence and also showed more than 90% identity with other teleosts. The C-terminal domain with hydrophobic amino acids forms a tunnel structure that is believed to transport cholesterol to mitochondria is also highly conserved. Our data further supports that the primary structure of StAR, particularly in the functional domain is highly conserved throughout vertebrates. Northern blot analysis identified a single transcript of about 1.2 kb in catfish ovary. Though multiple transcripts have been identified in humans (Sugawara *et al*., 1995) and

some in trout (Kusakabe *et al*., 2002), the size of StAR protein seems to be more or less similar in all the vertebrates except for the human splice variant.

We identified a single transcript in ovary and multiple transcripts in testis. Occurrence of multiple transcripts in testis could be either due to the presence of different UTR variants or un- processed mRNAs as described in earlier reports (Kusakabe *et al*., 2002, Li *et al*., 2003). Consistent with previous studies, using RT-PCR, *StAR* transcripts were found both in classical and non-classical steroidogenic tissues including ovary, testis, head kidney, liver and a faint signal was also observed in intestine and brain. Presence of high level of *StAR* transcripts in gonads and head kidney (equivalent to mammalian adrenal) is in agreement with the previous reports (Sugawara *et al*., 1995; Kusakabe *et al*., 2002). A low level of *StAR* transcript was also found in catfish brain, however presence of *StAR* in brain was already reported in rat and marmoset (Furukawa *et al*., 1998; Bauer *et al*., 2000). Co-localization of StAR with P450scc and 3β-hydroxysteroid dehydrogenase in brain indicates a role for StAR in neurosteroid synthesis (Furukawa *et al*., 1998). In addition to classical steroidogenic tissues, teleost *StAR* was also found to express in wide range of tissues including spleen, intestine, gill, skin (Kusakabe *et al*., 2002; Bauer *et al*., 2000; Li *et al*., 2003; Goetz *et al*., 2004) and muscle though the function of *StAR* in these tissues is yet to be established. However, in mammals, *StAR* expression is largely restricted to tissues capable of *de novo* steroid synthesis (Sugawara *et al*., 1995).

In the present study, using real-time RT-PCR, we found maximum expression in late vitellogenic stage of ovary. Previous studies using Northern blot hybridization documented similar kind of results in cod (Goetz *et al*., 2004) and trout (Kusakabe *et al*., 2002; Nakamura *et al*., 2005). In contrast, zebrafish *StAR* expression decreased with increase in follicle growth to maturation (Ings and Van der Kraak, 2006). Though we could not sample at many durations of different stages, a gradual increase in *StAR* transcript from early vitellogenesis to late vitellogenesis in trout is in accordance with circulating gonadotropins and presumed to be important for the production of estrogens/androgens at those times. In catfish testis, we noticed high expression in the preparatory phase while a low level in spawning phase. On the contrary, a 40 fold increase in *StAR* transcripts over the course of spermatogenesis, with peak values around late spermatogenesis and spermiation, is reported in trout (Kusakabe *et al*., 2002; Nakamura *et al*., 2005). The observed discrepancy of *StAR* expression among these species could be attributed to either sensitivity of the techniques used or to varied sampling or breeding patterns. However, high expression in the preparatory phase of catfish gonads that reflects active stage of spermatogenesis/vitellogenesis shows a positive correlation with gonadal cycle as of other steroidogeneic enzyme genes in catfish (Kumar *et al*., 2000; Chapter 2 & 4). In spite of the variations in expression pattern of *StAR* in different species of teleosts, change in the levels of *StAR* mRNA seems to be in harmony with the circulating gonadotropin levels indicating that *StAR* expression is under gonadotropin control.

Though there are reports on *StAR* expression following acute disturbance (Kusakabe *et al*., 2002), ACTH injection and change in salinity (Kim *et al*., 1997; Stocco, 2001; Li *et al*., 2003), reports on induction of *StAR* mRNA by gonadotropins in non-mammalian vertebrates are limited. In the present study, we demonstrate the induction of *StAR* mRNA by hCG both *in vitro* and *in vivo* during final oocyte maturation by real-time RT-PCR. Our observation shares similarity with that of zebrafish (Ings and Van der Kraak, 2006) and humans (Kiriakidou *et al*., 1996), wherein human *StAR* gene transcription was shown to be inducible by cAMP that is produced by LH-surge. On the contrary, ovarian follicles sampled at different time period of naturally ovulating trout showed minute expression before the onset of germinal vesicle breakdown (GVBD) and after GVBD while a several fold increment was observed at the time of ovulation and post ovulation (Kusakabe *et al*., 2002; Nakamura *et al*., 2005). In case of the Atlantic croaker, treatment of ovarian follicles with hCG did not alter *StAR* mRNA expression till 6 hours and a 17 fold increase was noticed at 24 hours (Nunez and Evans, 2007). Extensive sampling following hCG-treatment, *in vitro* and *in vivo* in catfish demonstrated the induction of *StAR* by gonadotropin in teleosts.

In conclusion, we isolated a cDNA encoding *StAR* from ovary of catfish which exhibited high level of sequence identity to other vertebrate *StAR* cDNAs. Spatial expression pattern together with expression in different stages of gonadal cycle are presented here. Elevation of *StAR* during hCG-induced oocyte maturation, *in vitro* and *in vivo* indicated a role for *StAR* in shift in steroidogenesis occurring in catfish ovarian

follicles prior to oocyte maturation or during final oocyte maturation. Based on our results, it is important to analyze the contribution of *StAR* in addition to sterodogenic enzyme genes or related transcription factors during meiotic maturation in teleosts.

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Chapter 1: Expression of *20β-HSD* and *P450c17* during hCG-induced *in vitro* oocyte maturation in snake head murrel, *Channa striatus*

To understand the molecular mechanism underlying shift in steroidogenesis, partial cDNAs encoding *20β-HSD* and *P450c17* were cloned from ovarian follicles of snake head murrel. Transcript level of both *20β-HSD* and *P450c17* increased from pre-vitellogenic stage to full-grown immature stage. A significant increase in transcript level of *20β-HSD* was found while *P450c17* noticed a minor increase in hCG-induced oocyte maturation, *in vitro*. These preliminary results tend to suggest the involvement of *P450c17* in addition to *20β-HSD* in shift in steroidogenesis. However, there was no obvious correlation between the transcript and protein levels of *P450c17* suggesting a possibility for the post-translational modifications. More in depth studies are necessary to confirm this notion. Owing to some technical difficulties with this species, further studies are continued in air-breathing catfish *Clarias gariepinus*.

Chapter 2: Evidences for the involvement of *20β-HSD* in final oocyte maturation

To implicate a role for *20β-HSD* in oocyte maturation more explicitly, *20β-HSD* cDNAs are cloned and characterized from gonads of catfish. *20β-HSD* cDNAs isolated from ovary and testis are 3'UTR variants. A significant difference in 20β-HSD/CR activity on both steroids and xenobiotics was found between the full-length and an N-

terminal deletion mutant recombinant protein expressed in *E. coli*. A positive correlation of *20β-HSD* expression was observed during ovarian and testicular cycles. An elevation in *20β-HSD* expression and activity was observed during hCG-induced oocyte maturation, *in vitro* and *in vivo*. Present comprehensive study provides new insights for the involvement of *20β-HSD* in oocyte maturation using chemical inhibitors and immunolocalization together with expression studies.

Chapter 3: Transcriptional regulation of *20β-HSD*: Role of *CREB*

Though several reports showed the involvement of *20β-HSD* in teleost oocyte maturation, transcriptional regulation has not been probed so far. A 2.0 kb upstream sequence from transcriptional start point of *20β-HSD* gene was isolated from catfish. *In silico* analysis demonstrated for the presence of several putative transcription factor binding sites including cAMP, xenobiotic, progesterone, androgen, estrogen, glucocorticoid response elements, AP-1, SP-1, GATA FOXL-1, SOX-5, SOX-9, Ad4BP/SF-1, OCT-1 etc. Luciferase reporter activities with progressive PCR based deletion mutants in CHO and HEK293 cells indicated that -435 region encompassing cAMP responsive element flanked with a CAAT and TATA boxes are important for basal promoter activity. cAMP altering drugs such as forskolin and IBMX enhanced the promoter activity suggesting the induction of expression by cAMP. A cDNA encoding cAMP responsive element binding protein (*CREB*) cloned from catfish ovarian follicles

seems to be very ancient among the vertebrate *CREB*s. Synergistic expression pattern of *CREB* with *20β-HSD* indicates that the latter is transcriptional regulated by the former.

Chapter 4: A role for *P450c17* during shift in steroidogenesis that occurs in ovarian follicles prior to oocyte maturation

Shift in steroidogenesis during oocyte maturation can also be regulated at the level of precursor steroid production and *P450c17* is the enzyme that produces precursor steroid, 17α-OHP. A cDNA encoding *P450c17* was isolated from ovarian follicles that is highly homologous to other teleost *P450c17-I* while it showed only about 40% homology to the recently identified novel *P450c17* (type-II) that lacks lyase activity. A positive correlation of *P450c17* expression and lyase/hydroxylase activity was found with ovarian cycle. A significant increase in *P450c17* expression was noticed during hCG-induced oocyte maturation, both *in vitro* and *in vivo*. However, there was no significant change either in protein level or in lyase/hydroxylase activity. These results suggest that the potentiation of *P450c17* in preparatory/pre-spawning phases might contribute to some extent for the shift in steroidogenesis. Though this study categorically demonstrated the expression and activity in oocyte maturation, *in vitro* and *in vivo*, regulation of lyase activity with reference to cytochrome b5 and P450 oxidoreductase is worthy for further analysis.

Chapter 5: A role for *StAR* during shift in steroidogenesis that occurs in ovarian follicles prior to oocyte maturation

StAR is a mitochondrial sterol transfer protein that regulates acute steroidogenesis in response to factors such as tropic hormones. Whether acute steroidogenesis has a role in shift in steroidogenesis is yet to be studied though there are reports cloning *StAR* cDNAs from few teleosts species. A cDNA encoding *StAR* was cloned from ovarian follicles of catfish. We present data on *StAR* expression in both ovarian and testicular cycles. In hCG-induced oocyte maturation, *in vitro* and *in vivo*, *StAR* transcripts significantly increased suggesting a role for *StAR* in steroidogenic shift in ovarian follicles that occurs prior to oocyte maturation.

In conclusion, the present study provides information on the induction of *20β-HSD* expression during oocyte maturation in *Channa striatus*. Immunolocalization, induction of *20β-HSD* expression and activity together with the inhibition of recombinant 20β-HSD activity as well as hCG-induced germinal vesicle breakdown by carbonyl reductase inhibitors provides new evidences for the involvement of *20β-HSD* in oocyte maturation using catfish model. Present thesis also examined the promoter elements of *20β-HSD* which provided new insights into its transcriptional regulation. In addition, a role for *P450c17* and *StAR* to the steroidogenic shift has also been proposed.

Research publications

1. **Sreenivasulu, G.,** Senthilkumaran, B., **2009.** Cloning, characterization of 20β-hydroxysteroid dehydrogenase from the gonads of air-breathing catfish (*Clarias gariepinus*) and insights for its involvement in final oocyte maturation. **Gene** (**Submitted**).

2. **Sreenivasulu, G.,** Senthilkumran, B., **2008.** A role for cytochrome P450 17α-hydroxylase/c17-20 lyase during shift in steroidogenesis occurring in ovarian follicles prior to oocyte maturation. **Journal of Steroid Biochemistry and Molecular Biology** (**In review**).

3. **Sreenivasulu, G**., Sridevi, P., Sahoo, P.K., Swapna, I., Ge, W., Kirubagaran, R., Dutta-Gupta, A., Senthilkumaran, B., **2008**. Cloning and expression of StAR during gonadal cycle and hCG-induced oocyte maturation of air-breathing catfish, *Clarias gariepinus*. **Comparative Biochemistry and Physiology B** (**In review**).

4. **Sreenivasulu, G.**, Swapna, I., Rasheeda, M.K., Ijiri, S., Adachi, S., Thangaraj, K., Senthilkumaran, B., **2005.** Expression of 20β-hydroxysteroid dehydrogenase and P450 17α-hydroxylase/c17-20 lyase during hCG-induced *in vitro* oocyte maturation in snake head murrel *Channa striatus*. **Fish Physiology and Biochemistry** 31, 227–230 (Springer, Holland).

Research publications by team work with members of Prof. B. Senthilkumaran's laboratory

5. Senthilkumaran, B., Sudhakumari, C.C., Wang, D.S., **Sreenivasulu, G.,** Kobayashi, T., Kobayashi, H.K., Yoshikuni, M., Nagahama, Y., **2008.** Novel forms of 3β-hydroxysteroid dehydrogenases from gonads of the Nile tilapia: Phylogenetic significance and expression during reproductive cycle. **Molecular and Cellular Endocrinology (In press).**

6. Swapna, I., Sudhakumari, C.C., Sakai, F., **Sreenivasulu, G.,** Kobayashi, T., Kagawa, H., Nagahama, Y., Senthilkumaran, B., **2008.** SbGnRH immunoreactivity in brain and pituitary of XX and XY Nile tilapia, *Oreochromis niloticus* during early development. **Journal of Experimental Zoology** 309A, 419-426 (Wiely-Blacewell, USA).

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8. Swapna, I., **Sreenivasulu, G.**, Rasheeda, M.K., Thangaraj, K., Kirubagaran, R., Okuzawa, K., Kagawa, H., Senthilkumaran, B., **2005.** Seabream GnRH: Partial cDNA cloning, localization and stage dependent expression in brain and ovary of snake head murrel, *Channa striatus*. **Fish Physiology and Biochemistry** 31, 157-161 (Springer, Holland).

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